The Determination of Sinigrin in *Brassica*, and Investigations into the Use of Allyl-Isothiocyanate as a Nematicide.

By
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ABSTRACT

The goal of this thesis was to study factors related to the development of *Brassica juncea* as a sustainable nematicide. *Brassica juncea* is characterized by the glycoside (glucosinolate) sinigrin. Various methods were developed for the determination of sinigrin in *Brassica juncea* tissue extracts. Sinigrin concentrations in plant tissues at various stages of growth were monitored. Sinigrin enzymatically breaks down into allyl-isothiocyanate (AITC). AITC is unstable in aqueous solution and degradation was studied in water and in soil. Finally, the toxicity of AITC against the root-lesion nematode (*Pratylenchus penetrans*) was determined.

A method was developed to extract sinigrin from whole *Brassica juncea* tissues. The optimal time of extraction with boiling phosphate buffer (0.7mM, pH=6.38) and methanol/water (70:30 v/v) solutions were both 25 minutes. Methanol/water extracted 13% greater amount of sinigrin than phosphate buffer solution. Degradation of sinigrin in boiling phosphate buffer solution (0.13%/minute) was similar to the loss of sinigrin during the extraction procedure. The loss of sinigrin from boiling methanol/water was estimated to be 0.01%/minute. *Brassica juncea* extract clean up was accomplished by an ion-pair solid phase extraction (SPE) method. The recovery of sinigrin was 92.6% and coextractive impurities were not detected in the cleaned up extract.

Several high performance liquid chromatography (HPLC) methods were developed for the determination of sinigrin. All the developed methods employed an isocratic mobile phase system with a low concentration of phosphate buffer solution, ammonium acetate solution or an ion-pair reagent solution. A step gradient system was also developed. The method involved preconditioning the analytical column with phosphate buffer solution and then switching the mobile phase to 100% water after sample injection.

Sinigrin and benzyl-glucosinolate were both studied by HPLC particle beam negative chemical ionization mass spectrometry (HPLC-PB-NCI-MS). Comparison of the mass spectra revealed the presence of fragments arising from the thioglucose moiety and glucosinolate side-chain.

Variation in the sinigrin concentration within *Brassica juncea* plants was studied (Domo and Cutlass cultivars). The sinigrin concentration in the top three leaves was studied during growth of each cultivar. For Cutlass, the minimum ($200\pm100\mu g/g$) and maximum ($1300\pm200\mu g/g$) concentrations were observed at the third and seventh week after planting, respectively. For Domo, the minimum ($190\pm70\mu g/g$) and maximum ($1100\pm400\mu g/g$) concentrations were observed at the fourth and eighth week after planting, respectively. The highest sinigrin concentration was observed in flower tissues $2050\pm90\mu g/g$ and $2300\pm100\mu g/g$ for Cutlass and Domo cultivars, respectively.

Physical properties of AITC were studied. The solubility of AITC in water was determined to be approximately $1290\mu g/ml$ at 24°C. An HPLC method was developed for the separation of degradation compounds from aqueous AITC sample solutions. Some of the degradation compounds identified have not been reported in the literature: allyl-thiourea, allyl-thiocyanate and diallyl-sulfide. In water, AITC degradation to diallyl-thiourea was favored at basic pH (9.07) and degradation to diallyl-sulfide was favored at acidic pH (4.97). It was necessary to amend the aqueous AITC sample solution with acetonitrile before injection into the HPLC system. The acetonitrile amendment considerably improved AITC recovery and the reproducibility of the results.

The half-life of aqueous AITC degradation at room temperature did not follow first-order kinetics. Beginning with a $1084\mu g/ml$ solution, the half-life was 633 hours. With an initial AITC concentration of $335\mu g/ml$ the half-life was 865 hours. At 35°C the half-life AITC was 76 ± 4 hours essentially independent of the

solution pH over the range of pH=4.97 to 9.07 (1000µg/ml). AITC degradation was also studied in soil at 35°C; after 24 hours approximately 75% of the initial AITC addition was unrecoverable by water extraction.

The EC_{50} of aqueous AITC against the root-lesion nematode (*Pratylenchus penetrans*) was determined to be approximately 20µg/ml at one hour exposure of the nematode to the test solution. The toxicological study was also performed with a myrosinase treated *Brassica juncea* extract. Myrosinase treatment of the *Brassica juncea* extract gave nearly quantitative conversion of sinigrin into AITC. The myrosinase treated extract was of the same efficacy as an aqueous AITC solution of equivalent concentration.

The work of this thesis was focused upon understanding parameters relevant to the development of *Brassica juncea* as a sustainable nematicide. The broad range of experiments were undertaken in support of a research priority at Agriculture and Agri-Food Canada.

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Special thanks are deserved by the staff and management at Agriculture and Agri-Food Canada. Mitch Pogoda helped with sampling *Brassica juncea* tissues and the clean up of extracts. Also, Mitch Pagoda was responsible for assessing nematode mortality to generate the EC_{50} value of allyl-isothiocyanate for the root-lesion nematode. Marta Hernandez was of assistance during the experiments related to the study of AITC degradation in aqueous solution. Sheridan Alder was of great help in acquiring the journal articles that were necessary for the preparation of this thesis. I would also like to thank the greenhouse staff for help with the growth of *Brassica juncea* plants.

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On our fourth wedding anniversary, this thesis is dedicated to my wife, Nina, and our daughter Ami. Without Nina's help and support this thesis would never have been completed.

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INTRODUCTION

1. Background

One research priority at Agriculture and Agri-Food Canada, Pest Management Research Centre (Vineland Station, Ontario) is the development of mustard (*Brassica juncea*) plants or plant parts as a pest control agent[1]. This research concern is also shared by the Ontario Pesticide Advisory Committee (Ministry of the Environment, Ontario). The Ontario Pesticide Advisory has suggested that the development of biological pest control methods be a pesticide research priority[1]. Below the literature relevant to the development of *Brassica juncea* as a nematicidal agent is presented.

2. Nematodes

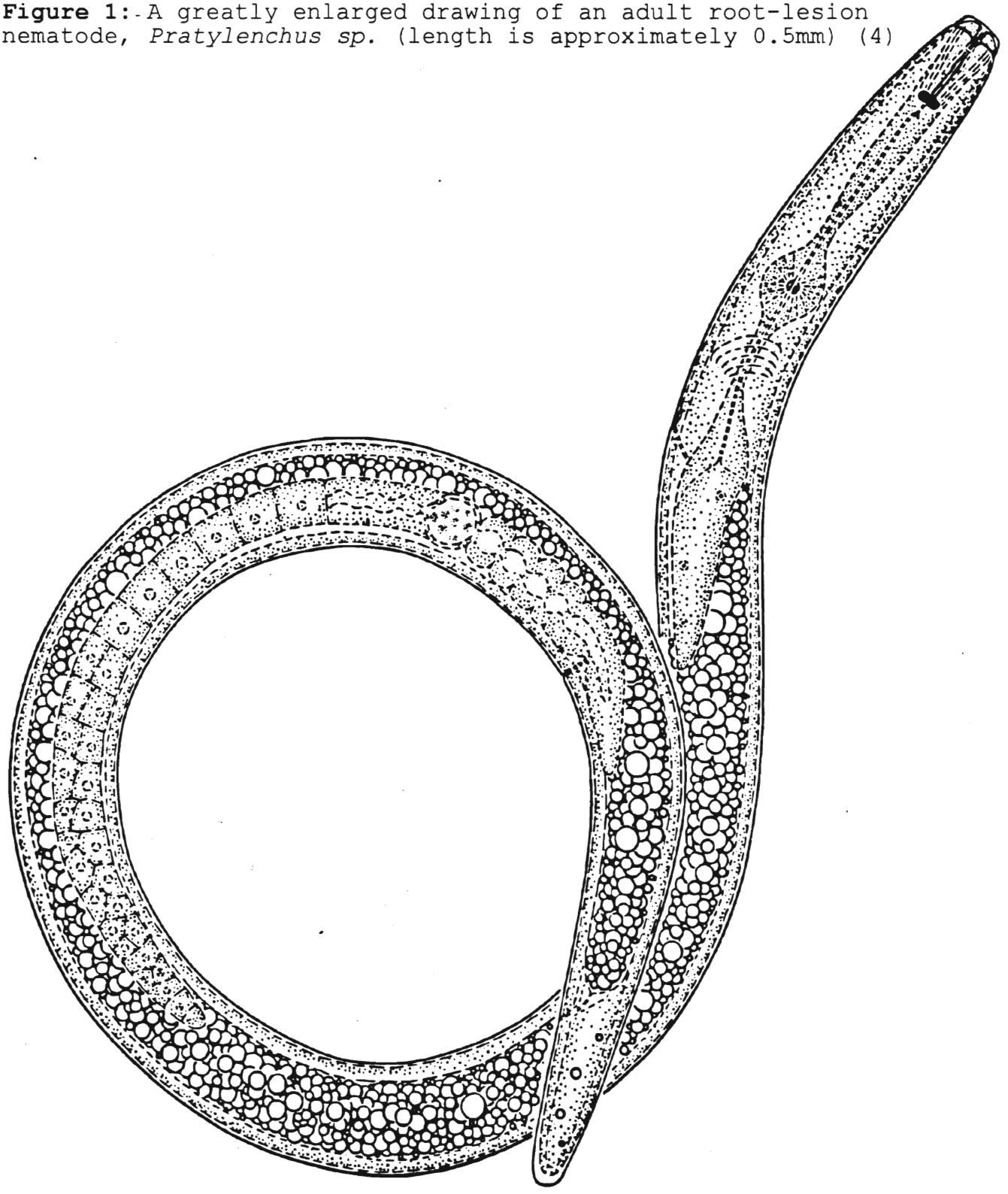
Nematodes are microscopic parasitic worms (**Figure 1**) that cause great economic losses in many crops. The most common nematodes in Ontario that would interfere with crop production are the root-lesion (*Pratylenchus sp.*) and the root-knot (*Meloidogyne sp.*) nematodes[2]. The root-knot nematode is considered the more destructive pest of the two[2].

1) Importance of control

Most of the human population relies on vegetables as a major source of nutrition[3]. Vegetable production is often hampered by nematode predation under all climatic conditions[3,4]. It has been estimated that the average economic loss for all crops by nematode infestation is approximately 11%[3].

The economic significance of nematode predation varies with the crop species considered. Some crops such as spinach and cabbage are tolerant to root-knot nematode infestation[3]. These plants may be stunted in the presence of high soil nematode populations but a saleable crop may still be harvested[3].

Figure 1: A greatly enlarged drawing of an adult root-lesion nematode, *Pratylenchus sp.* (length is approximately 0.5mm) (4)



The root-knot nematode is a significant pest of potato (in the Pacific Northwest)[5,6]. The nematode causes reduced tuber quality by stimulating surface wart formation and internal brown spots. Economic losses occur because the potatoes may be downgraded or completely rejected for sale[5].

Carrots are highly susceptible to deformation by the root-knot nematode. Damaged carrots are rejected for sale (**Appendix 1**). Carrots have a zero tolerance threshold for the presence of root-knot nematodes in the soil. If nematode juveniles are present in the soil a damage-producing situation already exists[3].

2) Chemical control of nematodes

The oldest method of nematode control is crop rotation[7]. Crop rotation is not the ideal solution for nematode control. Nematode populations quickly increase in the presence of a host crop and in its absence the population takes many years to decline[7]. Chemical control with nematicidal agents has been very successful in the control of nematode populations. Studies have estimated that nematicides have increased crop yields in the United States by 87%[8].

Control of soil nematode infestation is heavily dependant upon the use of fumigation. Soil fumigants are volatile compounds with moderate water solubility[7]. Application of fumigants often involves injection of the compounds into the soil. The compounds are distributed throughout the soil by diffusion in the gas phase[7]. Nematicidal fumigants such as 1,3-dichloropropene or metham sodium (sodium methyl-dithiocarbamate) are commonly employed[5,8,9,10]. Fumigants such as chloropicrin (CCl_3NO_2)[11], methyl-bromide and methyl-isothiocyanate (MITC) have also been used[8,10,12].

Soil fumigation costs approximately $1000 (Canadian) per hectare[2]. A low cost alternative to soil fumigation would allow nematode control measures to be extended to less expensive crops.

Most field crops, vegetables and some specialty crops are not valuable enough to warrant soil fumigation[2].

Alternatives to soil fumigants are actively being sought because of toxicological and environmental concerns. For example, methyl-bromide and chloropicrin are both highly toxic and volatile[10,11,12]. Often these compounds are applied by specially trained contractors[10]. The use of methyl-bromide is further complicated because the ground must be sealed with a plastic cover after application of the fumigant[10]. The release of halogenated hydrocarbons into the environment has become tightly controlled[13]. Studies have suggested that volatile halogenated organic compounds damage the stratospheric ozone layer[14,15,16,17]. Therefore, the use of fumigants such as methyl-bromide, chloropicrin, and 1,3-dichloropropene can be expected to be replaced with safer nematicidal agents in the future.

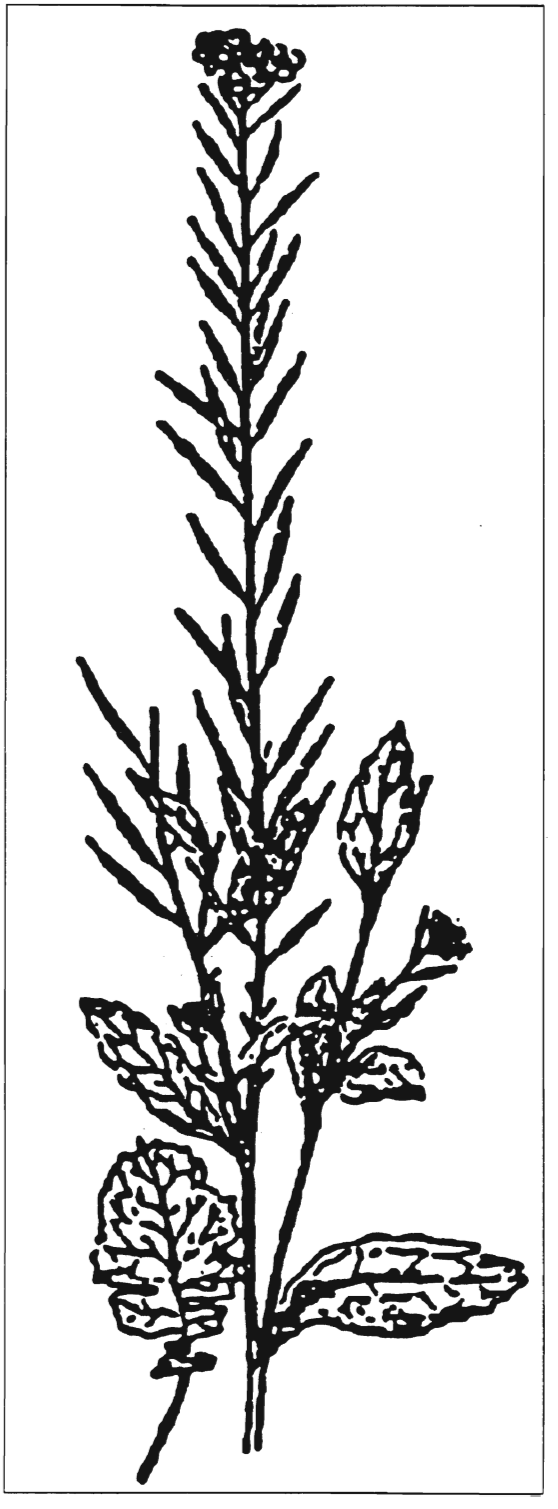
3. *Brassica juncea*

The plant genus *Brassica* is a member of the family *Cruciferae*[18]. Many common plants, such as broccoli, cauliflower, cabbage (*B. oleracea*), canola (*B. napus*) and mustard (*B. juncea*, **Figure 2**) are members of the genus *Brassica*[18,19]. Mustard (*Brassica juncea*) is an ancient cultivated plant used medicinally and as a condiment[20]. Mustard preparations are characterized by a pungent odor and a biting taste[20,21,22]. The plant is widely cultivated in Eastern Europe, Russia and the Orient[19,23,24]. *Brassica juncea* (cv. Cosson) can be found as a wild annual weed in Ontario fields[25].

1) Nematicidal properties

The process of growing a cover crop and turning that crop into the soil is known as green manuring[19,26]. Green manuring is an ancient practice that dates back before 1134 B.C.[19,26]. The incorporation of *Brassica sp.* tissue into nematode infested soil leads to a reduction of the nematode population[5,9,19,27,28,29]. Akhtar and Alam found that when a mixed crop of mustard (*Brassica juncea*)

Figure 2: A drawing of flowering *Brassica juncea* (mustard)



and potatoes were grown the nematode population in the soil was reduced[28]. The yield of the potato crop was increased by 44.2% (above the control)[28]. Singh et al. also found decreased nematode populations in *Brassica juncea* amended soils[27]. Further benefits of the use of *Brassica sp.* green manure are decreased weed emergence[30] and fungicidal activity[19,31]. The above studies suggest that *Brassica sp.* green manure has the potential to be developed into a sustainable substitute for the chemical control of nematodes, fungi and weeds.

The nematicidal activity of *Brassica sp.* has been attributed to the presence of a class of glucosides (glucosinolates, **Figure 3**) present in the plant tissues[32]. The nematicidal activity is known to derive from the products of glucosinolate enzymatic decomposition[32,33,34].

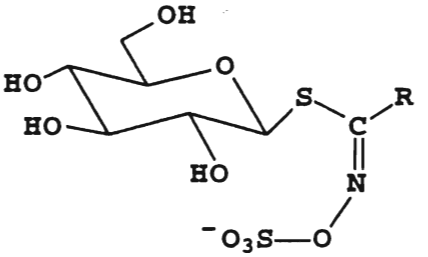
The mortality of the cyst nematode (*Heteroda schachtii*) in the presence of enzymatically degraded glucosinolates, was studied[32]. Of a series of glucosinolates studied, sinigrin (**Figure 3**) was the most active nematicidal glucosinolate[32]. Active glucosinolates are found in radish (*Raphanus sativus ssp. oleiformis*) roots. Radish is resistant to attack by the cyst nematode. The degree of nematode resistance of various radish cultivars is correlated to level of glucosinolates in the root system[32].

4. Glucosinolates (sinigrin)

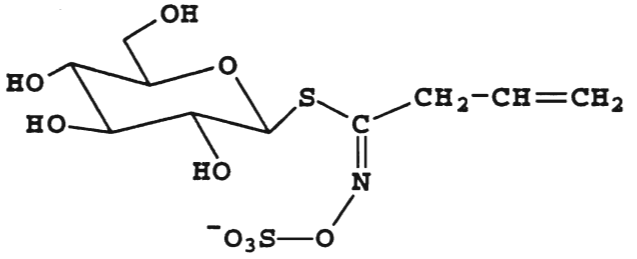
1) General properties

Glucosinolates are thioglucosides found widely in the plant family *Cruciferae*[23,35]. In 1839 a crystalline glucosinolate sinigrin (**Figure 3**) was first isolated (from *Brassica nigra*)[35,36]. The currently accepted general structure of glucosinolates was first proposed in 1897[36]. Ettlinger and Lundeen confirmed the correct structure of sinigrin in 1956[36]. The general glucosinolate structure (**Figure 3**) was determined to contain a thioglucose moiety and sulfonated oxime group linked to a side-chain (R)[36,37].

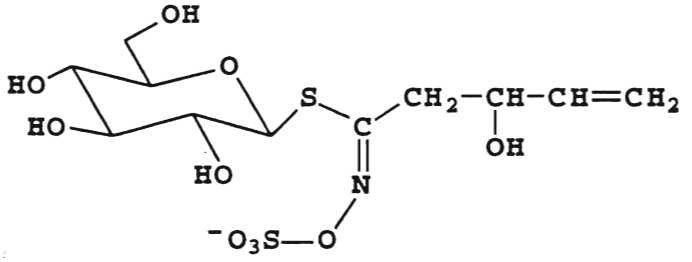
Figure 3: Structures, semi-systematic and trivial names of selected glucosinolates



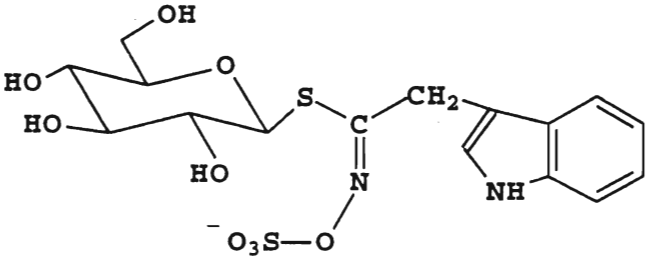
general glucosinolate structure



allyl-glucosinolate (sinigrin)



2-hydroxy-3-butenyl-glucosinolate (progoitrin)



3-indoylmethyl-glucosinolate (glucobrassicin)

The general structure of glucosinolates along with systematic, semi-systematic and trivial names for a few representatives is given in **Figure 3.** The semi-systematic names for glucosinolates rely upon the systematic chemical name of the side-chain used as a prefix before the word glucosinolate. For example, the semi-systematic name for sinigrin is allyl-glucosinolate. The systematic name for sinigrin is 1-thio-β-D-glucopyranose-1-[N-(sulfo-oxy)-3-butenimidate][38]. Throughout the rest of this thesis the semi-systematic nomenclature will be used for all glucosinolates except, allyl-glucosinolate which will be called sinigrin.

Sinigrin is quite stable in aqueous solution at ambient temperatures. The estimated activation energy for the hydrolysis of sinigrin in aqueous solution is 22.6kcal/mole[33]. MacLeod and Rossiter studied the stability of sinigrin and other glucosinolates in aqueous solution at 100°C[39]. After heating for one hour in aqueous solution 96.1% of the sinigrin was undegraded[39]. Further studies of sinigrin stability have supported the research of MacLeod and Rossiter[40,41].

Nearly all members of the family *Cruciferae* studied, have been shown to contain glucosinolates[23]. Approximately one hundred unique glucosinolates have been isolated[42]. Many glucosinolate structural types have been identified[46]. Short chain alkenyl-glucosinolates such as sinigrin (*Brassica juncea*)[23,42,43], 3-butenyl and 4-pentenyl-glucosinolate have been isolated (*Brassica napus*)[44]. Glucosinolates with 3-indolylmethyl side-chains have also been isolated: 3-indolylmethyl-glucosinolate, and 4-hydroxy-3-indolylmethyl-glucosinolate[45]. A homologous series of glucosinolates is also known[46]. A series of ω-(methylsulfinyl)alkyl-glucosinolates ($R=CH_3(S=O)[CH_2]_n$, n=3-6 and n=8-10)[23,47] have been identified from crucifers such as broccoli, cabbage, cauliflower and *Lesquerella fenleri*[48].

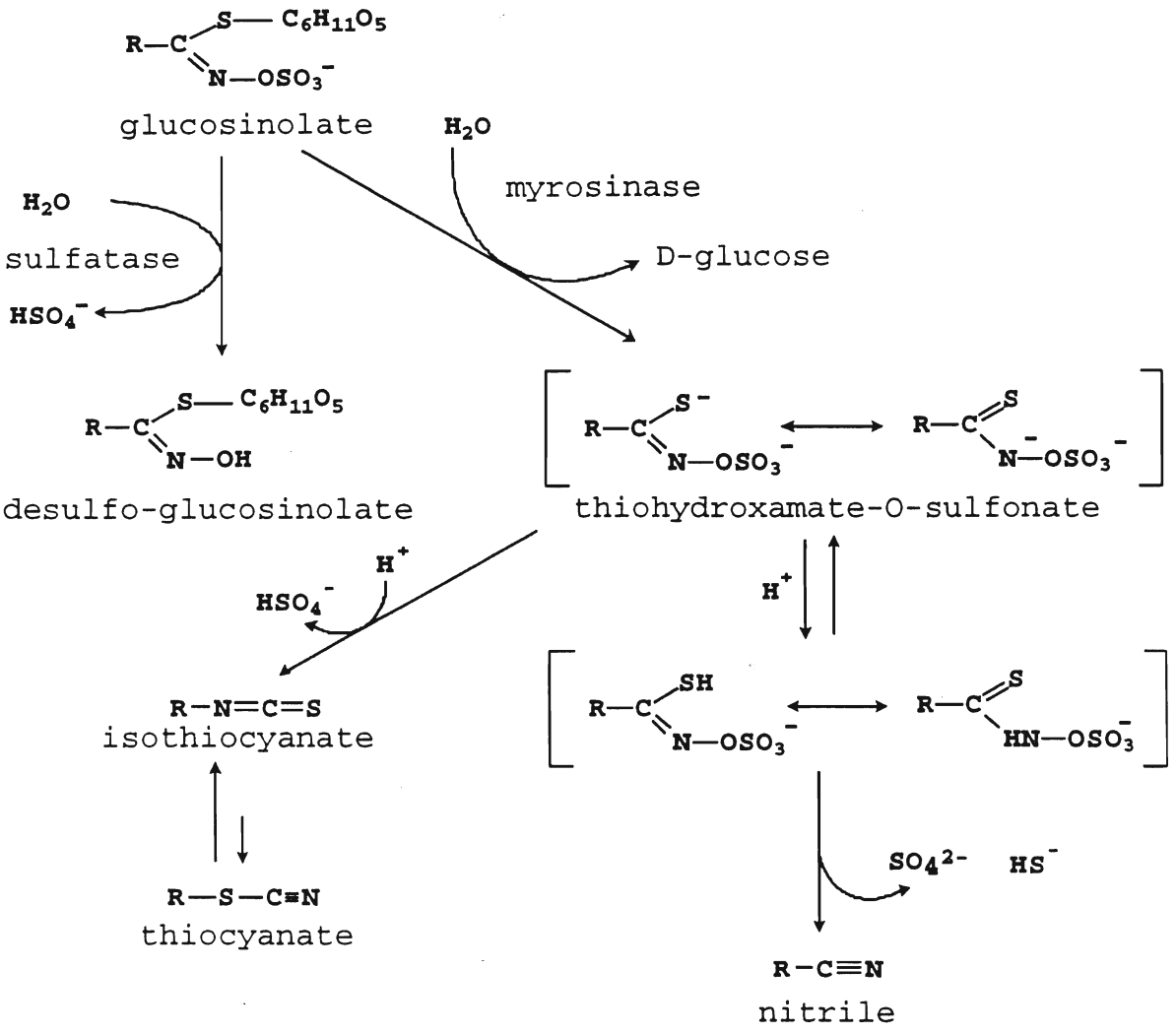
2) Degradation by Myrosinase

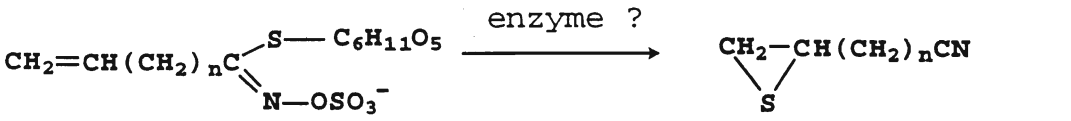
Glucosinolates are degraded by the enzyme myrosinase. The enzyme is known as a thioglucoside glucohydrolase. Myrosinase accepts a variety of glucosinolates with varying R-substituents[49]. Myrosinase cleaves only the thioglucoside linkage in glucosinolates to yield an unstable aglycone (thiohydroximate-O-sulfonate, **Figure 4**). The half-life of the aglycone derived from sinigrin is approximately 30 seconds in aqueous solution at 24°C[46].

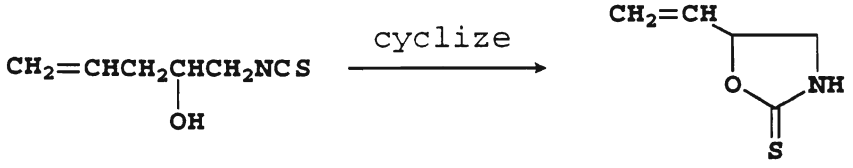
Studies have shown that the aglycone degrades in aqueous solution by two main mechanisms (**Figure 4**)[50,51]. The two major products of glucosinolate hydrolysis are isothiocyanates (R-N=C=S) and nitriles (R-CN). The aglycone may undergo a spontaneous Lossen rearrangement with concerted loss of sulfate to yield an isothiocyanate (**Figure 4**). The degradation of sinigrin was studied and allyl-isothiocyanate (AITC) formation was favoured under conditions more alkaline than pH=3.5[50,51]. Under acidic conditions the aglycone is protonated and cannot readily undergo Lossen rearrangement so, the nitrile is the predominant product (**Figure 4**)[51]. The nitrile is formed at the expense of the isothiocyanate. The nitrile degradation pathway is favored in the presence of certain metal ions, particularly Fe^{2+} [52,53]. Ferric (Fe^{3+}) ion appears to have no effect upon the isothiocyanate and nitrile pathways[50]. Exceptions to the above presented pathways have been noted. The conditions affecting glucosinolate hydrolysis and the nature of the myrosinase enzyme system has not been fully deduced[51,54].

Other myrosinase mediated glucosinolate degradation products have been detected[24]. Allyl-thiocyanate has been reported as a product of the myrosinase degradation of sinigrin but the conditions causing the formation of allyl-thiocyanate have not been deduced[21,50]. It has been suggested that alkyl-thiocyanate formation may be enzymatically mediated[21,49]. There is, however, evidence that in aqueous solutions allyl-thiocyanate may be in equilibrium with AITC[55,56,57,58]. 1-cyanoepithioalkanes arise from alkenyl-glucosinolates (**Figure 4**)[59]. It has been suggested that the

Figure 4: Enzymatic and chemical degradation of glucosinolates







rearrangement of the aglycone to 1-cyanoepithioalkene may be mediated by an enzyme[21,49,50].

Glucosinolates with β-hydroxyl-substituent side-chains (R) yield unstable hydroxy-isothiocyanates that cyclize (non-enzymatically) to form oxazolidinethiones. 5-vinyloxazolidine-2-thione (goitrin, **Figure 4**) is a breakdown product of 2-hydroxy-3-butenyl-glucosinolate (progoitrin, **Figure 3**)[60,61,62]. Goitrin is known to display antithyroid activity and can cause goiter[60,63,64]. Some cases of goiter are associated with diets rich in 2-hydroxy-3-butenyl-glucosinolate containing foods (such as cabbage, chard, rape and turnip)[60].

All plant species that produce glucosinolates also contain the enzyme myrosinase[65,66]. The plant myrosinase is found mainly within the family *Cruciferae*[67], although the enzyme also occurs in fungi[68,69], bacteria[68] and mammalian tissues[70]. Myrosinase and glucosinolates are segregated in the plant tissue. Studies have shown that myrosinase is confined to special cells called myrosin cells[71]. Glucosinolates are located in the vacuole of the cell[65]. Disruption of the plant cell activates the enzyme myrosinase and this results in isothiocyanates being released from glucosinolates. The hydrolysis of glucosinolates by myrosinase occurs very fast; 90% within one minute[22]. This arrangement has been called the "mustard oil bomb" and has been suggested to be an insecticidal, bactericidal and fungicidal defence mechanism[65,71].

3) Extraction from *Brassica sp.*

A review of the literature revealed that many different and often arbitrary methods for the extraction of glucosinolates have been employed. The critical literature regarding the extraction of glucosinolates from *Brassica sp.* tissues is presented in **Table 1.** Reports relevant to the extraction of sinigrin from *Brassica juncea* will be emphasized.

The use of boiling aqueous alcohol solvents is widely used for the extraction of glucosinolates from *Brassica sp.* tissues. Briffaud and Berot studied the extraction of rapeseed (*Brassica napus* and *B. campestris*) meal with various alcohols and water-alcohol mixtures (**Table 1,** #3). They found that at room temperature the degree of glucosinolate extraction increased with longer chain alcohols[72]. Buchner and Thies studied aqueous methanol glucosinolate extraction from rapeseed meal (**Table 1,** #4-8)[73]. A single 100% methanol extraction (10 minutes) at 60°C gave a low yield of total glucosinolates (72%)[73]. Other workers have used single 70% methanol extractions of rapeseed meal[74,75,76]. Bodnaryk and Palaniswamy used a single boiling 80% methanol extraction (5.0 minutes) for the extraction of glucosinolates from *Brassica juncea* cotyledons (**Table 1,** #2)[77].

Buchner and Thies reported maximal glucosinolate recovery was accomplished by two extractions (10 minutes) with boiling 70% methanol/water (**Table 1,** #6)[73]. Extraction times greater than 20 minutes caused a degradation of 4-hydroxy-indolylmethyl-glucosinolate[73]. Betz and Fox extracted *Brassica oleracea* fleshly tissue with two aqueous 70% methanol extractions (**Table 1,** #1)[78]. Sang et al. studied the glucosinolate contents of various *Brassica sp.* tissues, including *Brassica juncea* (**Table 1,** #19, 20)[44]. Sang et al. decided to use two different extraction methods. Aqueous methanol was used to extract green and fleshy tissue and water was used to extract the seed meal[44].

Boiling water has also been effectively used for the extraction of glucosinolates from rapeseed meal[36,79,80]. The official method of glucosinolate determination by the Canadian Grain Commission employs aqueous extraction of Canola (*Brassica napus*) and rapeseed meals[81]. Kozlowska et al. studied the aqueous extraction of glucosinolates from rapeseed meal (**Table 1,** #10-14)[80]. Kozlowska noted that a single aqueous extraction of the rapeseed meal essentially removed all glucosinolates (**Table 1,** #10). Studies have revealed that extraction with basic aqueous solution causes substantial glucosinolate degradation (**Table 1,** #12, 21)[80,82,83].

Table 1: Selected procedures used for the extraction of glucosinolates from *Brassica sp.* tissues.

#	Author(s)	GSN	Plant	Tissue	Solvent	Ratio g/ml	Means	Time (min)
1	Betz, Fox[78]	S-I	*Brassica oleracea*	freeze dried tissue	1) 70%MeOH 2) 70%MeOH	0.05	1) boil 2) wash	1) 2.5 min 2) 2x
2	Bodnaryk, Palaniswamy[77]	S,I	*Brassica juncea*	whole cotyledons	80%MeOH	0.05	boil	5min
3	Briffaud, Berot[72]	ns	*B. napus*	seed flour	water/var alcohols	0.10	stir	30min
4	Buchner, Thies[73]	S-I	*B. napus* *B. camp.*	seed flour	MeOH	0.10	s-l ext 1x (60°C)	10min
5	Buchner, Thies[73]	S-I	*B. napus* *B. camp.*	seed flour	70%MeOH	0.10	s-l ext 1x (60°C)	10min
6	Buchner, Thies[73]	S-I	*B. napus* *B. camp.*	seed flour	70%MeOH	0.10	s-l ext 2x (60°C)	10min 2x
7	Buchner, Thies[73]	S-I	*B. napus* *B. camp.*	seed flour	70%MeOH	0.10	s-l ext 2x (75°C)	5min 2x
8	Buchner, Thies[73]	S-I	*B. napus* *B. camp.*	seed flour	1) 70%MeOH 2) water	0.10	1) s-l ext (75°C) 2) s-l ext (75°C)	1) 2min 2) 8min, 10min
9	Kershaw, Johnstone[84]	ns	*B. napus* *B. camp.*	seed flour	1) heat 2) var %MeOH/ water	0.10	1) heat (150°C) 2) boil	1) 1-2 min 2) 20min
10	Kozlowska et al.[80]	ns	*B. napus* *B. camp.*	seed flour	water	0.20	stir	14hr

#	Author(s)	GSN	Plant	Tissue	Solvent	Ratio g/ml	Means	Time (min)
11	Kozlowska et al.[80]	ns	*B. napus* *B. camp.*	seed flour	1)water 2)water	0.20	1)boil 2)stir	1) 1.1hr 2) 14hr
12	Kozlowska et al.[80]	ns	*B. napus* *B. camp.*	whole seed	0.1N NaOH	0.20	stir	1.0hr
13	Kozlowska et al.[80]	ns	*B. napus* *B. camp.*	whole seed	1)water 2)0.1N NaOH	0.20	1) boil 2) stir	1) 1.1hr 2) 14hr
14	Kozlowska et al.[80]	ns	*B. napus* *B. camp.*	whole seed	1)water 2)70%EtOH 0.1N NaOH	0.20	1) boil 2) stir	1) 1.1hr 2) 14hr
15	Quinsac,[74] Ribaillier	P,H	*B. napus*	seed flour	water	0.02	boil	10min
16	Quinsac,[74] Ribaillier	P,H	*B. napus*	seed flour	5mM 2-ME/ 1mM EDTA	0.02	boil	10min
17	Quinsac,[74] Ribaillier	P,H	*B. napus*	seed flour	70%MeOH	0.02	boil	10min
18	Quinsac,[74] Ribaillier	P,H	*B. napus*	seed flour	70%MeOH	0.02	boil 2x	5min 2x
19	Sang et al.[44]	var (S)	*Brassica juncea* etc.	frozen ground root leaf	1)MeOH 2)75%MeOH	0.20	1) boil 2) boil	1) 3min 2) 3min
20	Sang et al.[44]	var (S)	*Brassica juncea* etc.	seed flour	1)heat 2)water	0.05	1)heat (100°C) 2) boil	1) 3min 2) 5min
21	Shahidi, Gabon[82,83]	P-I	*Brassica juncea* *B. napus* *B. camp.*	seed flour	1)10%NH_3 (w/w)/ 95%MeOH 2)Hexane 3)MeOH	0.15	1) blend 2)stand 3)wash	1) 2min 2) 15min 3) 3x

Table 1 abbreviations:

ns = not specified
var = various
s-l ext = solid-liquid extraction
2-ME = 2-mercapto-ethanol

GSN = glucosinolates
H = 4-hydroxy-3-indolylmethyl-glucosinolate
I = 3-indolylmethyl-glucosinolate
P = 2-hydroxy-3-butenyl-glucosinolate
S = Sinigrin
P-I = various glucosinolates including P and I
S-I = various glucosinolates including S and I

Ratio g/ml = grams of tissue per ml of solvent

Solvent = all solvents are aqueous solutions (v/v) unless otherwise stated

Kershaw and Johnstone compared the extraction of glucosinolates from rapeseed meal (after an initial heat treatment) by water and aqueous methanol solutions (**Table 1,** #9)[84]. Their results suggest that both water and aqueous methanol after repetitive extractions are of similar extractive efficiency[84].

Quinsac and Ribaillier studied the extraction of glucosinolates from ground rapeseed with a variety of solvents (**Table 1,** #15-18)[74]. They studied extraction by 70% aqueous methanol, water and a 5mM aqueous solution of 2-mercapto-ethanol with 1mM ethylenediamine-tetraacetic acid (EDTA)[74]. Water was more effective than methanol for the recovery of 4-hydroxy-indolylmethyl-glucosinolate from the rapeseed meal[74]. 2-mercapto-ethanol solution should increase the yield of 4-hydroxy-indolylmethyl-glucosinolate by inhibiting degradation but, experimentally recovery was poor[74].

Sosulski et al. noticed that glucosinolates may be removed from whole *Brassica sp.* seeds via diffusion extraction[85]. Diffusion extraction is based upon the observation that low molecular weight glucosinolates will pass through seed membranes, but larger molecules such as proteins and triglycerides will be retained[85]. Other studies have confirmed that diffusion extraction is effective at removing glucosinolates from whole *Brassica sp.* seeds (**Table 1,** #12-14)[80].

A search of the literature revealed that both water and aqueous alcohol solutions are used to extract glucosinolates from *Brassica sp.* tissues. Tissue to solvent ratios of 0.02g/ml to 0.20g/ml have been used (**Table 1**)[74,80]. Potentially, diffusion extraction may be used to extract glucosinolates from fleshy tissue (leaf, flower and stem). The literature would benefit from a systematic study of the diffusion extraction (with water and aqueous methanol solvents) of fleshy *Brassica juncea* tissue.

4) Clean up of *Brassica sp.* extracts

The *Brassica sp.* extract matrix contains many components that can adversely affect various analytical techniques. Phenolic compounds[86,87], lipids, carbohydrates and proteins have been extracted from rapeseed meal by alcohol/water mixtures[72,75]. Sinapine (sinapic acid choline ester) is a phenolic ester that occurs at high concentrations in *Cruciferae* seeds[88,89]. Sinapine has been identified as a possible interference in the HPLC determination of glucosinolates from rapeseed extracts[75]. Proteins in the sample can have a detrimental effect upon high performance liquid chromatography (HPLC) reversed-phase packing materials. Proteins may be precipitated, denatured and adsorbed onto the HPLC packing material leading to increased back pressure and altered chromatographic parameters[88,89].

Ion exchange is a standard method of isolating glucosinolates from *Brassica sp.* extracts[21,76,79,90]. Commonly used ion exchange materials are the crosslinked polysaccharide anion exchange resins based upon dextran (Sephadex A-25)[21,76,91] or cellulose (Ecteola)[76,92,93]. The aqueous extract is loaded onto a column of the anion exchange resin and interfering compounds such as carbohydrates are removed with a water wash[21]. The glucosinolates may then be isolated in high purity by washing the column with a buffer solution[21]. In many published methods the intact glucosinolates are not directly isolated. Instead, the glucosinolates in the ion exchange column are desulfated, by the action of sulfatase (**Figure 4**), and the desulfo-glucosinolates are then eluted[21,79,94,95,96]. Before ion exchange clean up lead and barium acetate is often added to the extract to precipitate sulfate ion[36,73] which would otherwise inhibit sulfatase[21]. Glucosinolate losses of approximately 10% have been observed with lead and barium acetate treatments[73]. Losses of as high as 20% have been observed for 1-methoxy-3-indolylmethyl-glucosinolate[73].

Solid phase extraction (SPE) is a widely used technique of liquid sample cleanup for HPLC[97]. SPE involves the concentration of

trace analytes from a liquid onto a sorbent packing material. Reversed-phase (C_8, C_{18})[98] or normal-phase (diol, nitrile)[98] silica-based sorbents are widely used SPE packing materials[97]. The packing material is usually supplied in a medical-grade polypropylene syringe barrel[97]. After concentration the analytes may be desorbed from the packing material by a suitable solvent[98]. Recently, Betz and Fox applied SPE (reversed-phase (C_{18}) silica-based) to the isolation of glucosinolates from aqueous *Brassica sp.* extracts[78]. The sorbent was preconditioned with tetrabutyl-ammonium sulfate (TBAS) to increase glucosinolate retention on the cartridge. The column was washed with water to remove impurities. The glucosinolates were then eluted with 55% methanol/water[78].

5) Methods of determination

The determination of glucosinolates diverges into two general strategies; gas chromatography (GC) and HPLC. Glucosinolates may be analyzed directly or the compounds may be desulfated (**Figure 4**) and appropriately derivatized before analysis. Other methods developed for the determination of glucosinolates will also be briefly considered.

i) Liquid chromatography

HPLC is well suited to the separation of water soluble and nonvolatile analytes such as glucosinolates. Glucosinolates have been analyzed by HPLC in both the intact forms and as their desulfo-derivatives (**Figure 4**).

The analytical method currently recommended by the Canadian Grain Commission for the determination of glucosinolates, in Canola and rapeseed, relies on reversed-phase HPLC (RP-HPLC) of the desulfo-derivatives[81]. RP-HPLC analysis of desulfo-glucosinolates is also a widely accepted analytical method in Europe (Commission of the European Communities)[99]. An aqueous acetonitrile gradient mobile phase is commonly employed[76,81,94,99-103]. Octadecyldimethyl-bonded silica (ODS, C_{18}) is the stationary phase of choice[76,81,94,99-103].

The desulfo-glucosinolates are detected by ultraviolet (UV) absorption at approximately 228nm (226nm[102,103] to 230nm[94]).

A drawback to glucosinolate analysis via the desulfo-derivative is in the preparation procedure for the derivative[76,99]. Glucosinolates are generally desulfated during the ion exchange clean up procedure (as previously described)[76,81,94,99-103]. Unfortunately, the method is time consuming and desulfation times requiring from three hours to 25 hours have been reported[76,99]. It should be noted that desulfation conditions (enzyme activity, temperature and pH) must be carefully controlled[76,99]. Losses of 4-hydroxyl-3-indolyl-gluconsinolate by the method have been noted[76].

Ionic compounds in a RP-HPLC system are usually eluted with the dead volume of the column[104]. It is possible to alter the chromatographic retention of ionic compounds by introducing ionic long-chain alkyl compounds into the HPLC mobile phase[105]. Retention of ionic compounds on alkyl-modified silica can be increased considerably by adding a lipophilic counter ion to the aqueous mobile phase. Liquid chromatography (LC) that exploits the use of long-chain alkyl counter ions in the mobile phase is called ion-pair chromatography. The counter ion added is called the ion-pair reagent. Addition of the ion-pair reagent alters the retention of the ionic compounds while not significantly affecting the retention of the nonionic compounds[105]. Ion-pair chromatography separations are highly dependent upon the pH of the mobile phase[106,107], the column temperature[106,107] and the nature and concentration of the ion-pair reagent in the mobile phase[108].

Ion-pair chromatography has been applied to the separation of sulfonic acid derivatives[104,109,110]. Tetraalkyl-ammonium salts[104,109], trialkyl-amines[111] or inorganic salts ($NaClO_4$)[104] have been used as ion-pair reagents. As previously stated, glucosinolates are sulfonic acid derivatives (**Figure 3**). Ion-pair chromatography has been successfully used for the separation of glucosinolates. Generally, ion-pair separations of glucosinolates are performed on ODS stationary phases. The most commonly used ion-pair reagent is

tetraheptyl-ammonium bromide (THAB)[74,92,93,112] at a concentration of 1.5mM to 5mM. Betz and Fox used TBAS (5mM) as the ion-pair reagent[78]. Helboe et al. found that sinigrin retention increases dramatically as the alkyl chain length of the ion-pair reagent increases[92]. Under identical conditions the retention of sinigrin is increased three fold by substituting tetraoctyl-ammonium bromide for THAB[92]. The ion-pair reagent is often prepared in phosphate buffer solution (10mM, pH=7)[74,92,93,112]. Either acetonitrile[74,112] or methanol[73,92,93] has been employed as an organic modifier in the mobile phase. The most common method of analyte detection was UV absorption at 235nm.

Both ammonium acetate[113,114] and phosphate buffer[113] mobile phases have been used with RP-HPLC for the determination of glucosinolates. Bjorkqvist and Hase found that glucosinolate retention, in a RP-HPLC system, was increased by using buffer mobile phases[113]. Retention increased with greater buffer salt concentrations in the mobile phase. But, the effect of increased salt concentration was less pronounced beyond approximately 0.1M[113]. An ammonium acetate buffer mobile phase was found to give sharper peaks than an equal concentration of phosphate buffer solution[113]. Compared with ion-pair chromatography methods, less organic modifier (acetonitrile) was required to elute the glucosinolates. Bjorkqvist and Hase used C_{18} (ODS) silica column as the reversed-phase stationary material. As with the ion-pair chromatography methods previously described, glucosinolates were detected by UV absorption at 235nm[113].

ii) Gas chromatography

Glucosinolates are not amenable to GC analysis in the intact form[41]. Lack of volatility and heat lability of the parent glucoside causes them to be unsuitable for GC analysis without derivatization. Studies have shown that glucosinolates can chemically degrade at column (GC) temperatures as low as 125°C to yield traces of the corresponding nitrile[41]. Temperatures as high as 200°C are necessary before the corresponding isothiocyanates are

formed but the yield is only 50%[41].

Trimethylsilyl (TMS) derivatization of the glucosinolate renders the compound sufficiently volatile so that separation by GC is possible. During the trimethylsilylation reaction glucosinolates undergo desulfation[115]. Therefore, trimethylsilylation of glucosinolates gives per(TMS)desulfo-glucosinolates. Many analytical procedures have been developed which employ GC analysis of per(TMS)desulfo-glucosinolates[79,95,115-119]. Desulfo-glucosinolates are the biosynthetic precursors for glucosinolates[21,120]. Desulfo-glucosinolates present before derivatization may interfere with the GC determination of glucosinolates. The derivatization reaction is another problematical aspect of the GC determination of glucosinolates. A series of unexpected products have been observed after the TMS derivatization of ω-(methylsulfinyl)alkyl-glucosinolates[21,118] and 1-methoxy-3-indolyl-glucosinolate[116].

iii) Mass spectrometry

A variety of mass spectrometry (MS) experiments have been applied to the identification of glucosinolates and desulfo-glucosinolates[121]. Electron impact (EI) ionization is a "hard" ionization technique in which a beam of energetic electrons (~70eV) bombards the sample[122]. The ionized sample molecules contain a high degree of internal energy and often fragment before they leave the ion source[122]. As expected, the EI-MS of per(TMS)desulfo-glucosinolates are characterized by a great deal of fragmentation[123,124]. Often, the molecular ion ($M^{+\cdot}$) is nonexistent and the base peak is the TMS fragment (m/z=73, $(CH_3)_3Si^+$)[123].

Chemical ionization (CI) is a "soft" ionization technique[122]. A reagent gas is added to the ionization chamber at a low pressure. The reagent gas is ionized by an EI mechanism. The ionized reagent gas then ionizes the sample by ion-molecule reactions. Often with the proper choice of reagent gas the molecular ion can be formed[122]. Positive ion CI (PCI) MS of per(TMS)desulfo-glucosinolates with methane[118] or isobutane[123] generally give more diagnostically useful

mass spectra than EI. The observed base peak is the protonated nitrile ($RCNH^+$)[118]. PCI-MS of glucosinolates has also been studied[124]. Glucosinolates also give $RCNH^+$ as the base peak when using methane or isobutane as the reagent gas[124]. When ammonia is used as the reagent gas the base peak is a glucose derived fragment (m/z=180), aglycone-ammonia adducts are also observed[124].

Negative ion chemical ionization (NCI) MS, of per(TMS)-desulfo-glucosinolates, is ten times more sensitive than PCI-MS[119]. Usually, a thioglucose derived fragment (m/z=140, $C_6H_5O_2S^-$) is the base peak under NCI conditions[119]. Of nearly the same intensity is a diagnostically useful fragment arising from α-cleavage of the thioether bond with charge retention on the side chain fragment ($TMS-ON=C(R)S^-$)[119].

Fast atom bombardment (FAB) is a "soft" ionization technique[125]. FAB-MS has been highly successful for the direct analysis of ionic, involatile and polar compounds[125]. Essentially, the sample is prepared in a low volatility matrix such as glycerol[125]. The sample and matrix are then bombarded with a fast heavy atom beam (such as Ar or Xe) causing the analyte to be sputtered into the gas phase[125]. FAB-MS has been applied to the determination of glucosinolates[114,121,125-127]. Positive ion FAB-MS of glucosinolates is characterized by intense $[M_a+C+H]^+$ and $[M_a+2C]^+$ peaks (where M_a is the glucosinolate anion and C is a cation)[121,125-127]. Typical fragmentation involves the loss of SO_3, thioglucose and glucose[126,127]. Positive ion FAB-MS of desulfo-glucosinolates is characterized by an intense $[M+H]^+$ peak with fragments arising from loss of thioglucose and glucose[128]. Negative ion FAB-MS of glucosinolates gives the molecular anion $M_a^{-\cdot}$ as the major peak[114,121,126,127].

Various techniques have been developed which allow HPLC to be interfaced with MS[129], some of these methods have been applied to the determination of glucosinolates[114,130-132]. Recently, the HPLC separation of glucosinolates has been interfaced with negative ion FAB-MS (continuous-flow frit FAB-MS)[114]. Thermospray (TSP) has also

been used to interface the HPLC separation desulfo-glucosinolates[130,131,132] with MS. In the positive ion mode the TSP-HPLC-MS spectra of desulfo-glucosinolates are similar to positive ion FAB-MS[121]. The protonated molecular ion $[M+H]^+$ is low in intensity[132] and the base peak is often the aglycone fragment[130] $[RC=NOH]^+$.

Particle-beam (PB) is another technique used to interface HPLC with MS[129]. HPLC-PB-MS has been used for a variety of analytes including PAHs[133] and amino acids[134]. With the PB interface, the column eluate is nebulized and dispersed as a fine mist[129]. The aerosol then passes through a desolvation chamber where the analytes condense to solid submicrometre sized particles[129]. Solvent, particles and a helium carrier gas are rapidly expanded from a small nozzle into a low pressure chamber[129]. The solute particles gain high momentum and form a linear beam while the other components are pumped away[129]. The particle beam is then transferred to the source of the mass spectrometer[129]. PB-HPLC-MS may be useful for glucosinolate determination. Of particular interest would be the PB-HPLC-MS study of glucosinolates under NCI conditions. NCI-PB-HPLC-MS can be expected to be a sensitive method with diagnostically useful fragmentation advantages.

iv) Miscellaneous methods

Many other analytical techniques have been used for the determination of glucosinolates. Enzyme-linked immunosorbent assay (ELISA), constructed from anti-sinigrin polyclonal antibodies, has been developed for alkenyl-glucosinolates[135]. The method is inexpensive and sensitive but aromatic glucosinolates are poorly detected[135]. Another inexpensive and rapid assay relies upon the determination of enzymatically released glucose. The action of myrosinase upon glucosinolates releases glucose that may be detected with clinical glucose sticks[136] or by the hexokinase colorimetric assay[84,137]. Glucosinolates have also been determined by near-infrared reflectance[138], x-ray fluorescence[112,138], H^1/C^{13}NMR[21,115] and as the palladium-glucosinolate complex[139]. Recently, micellar

electro-kinetic capillary chromatography (MECC) has been applied to separation of glucosinolates[140,141]. MECC is a high resolution electrophoretic technique that employs a surfactant/ion-pair reagent in the mobile phase[140,141].

5. Allyl-isothiocyanate

Allyl-isothiocyanate (3-isothiocyanato-1-propene or AITC) is commonly known as mustard oil. AITC is a product of enzymatic (myrosinase) decomposition of the glucosinolate sinigrin[46]. It is a strong pungent[142] volatile oil. AITC is a lacrymator vesicant and a suspected cancer causing agent[143]. Toxicological studies in rat revealed that the oral LD_{50} (50% lethal dose) is 339mg/kg (approximately 4.5 times the toxicity of vanillin)[144]. Studies have shown that AITC is a useful biocide against wireworm (*Limonius californicus*)[145], yeast (*Saccharomyces cerevisiae*)[146] and other microorganisms[147]. AITC is a widely used seasoning in the food industry. For example, AITC is used at the level of 0.50µg/g in ice cream to as high as 87µg/g in processed meats[148].

1) Nematicidal properties

The nematicidal properties of alkyl-isothiocyanates have been recognized since 1939[10]. The mechanism by which alkyl-isothiocyanates display nematicidal action has not been fully deduced. It is probable that the alkyl-isothiocyanates denature key enzymatic systems in the nematode. AITC is a protein denaturant by the observed cleavage of disulfide bonds in cystine[56,57] $[SCH_2CH(NH_2)CO_2H]_2$, insulin[56] and oxidized glutathione (GSSG)[56,149]. Studies have suggested that isothiocyanates may exert toxic action by reacting with the free thiol groups of enzymes[8,150]. Phenyl-isothiocyanate is a well-known reagent used in the determination of amino acid sequences by the Edman degradation[151,152]. Phenyl-isothiocyanate is a strong electrophillic reagent that reacts with the terminal amino group of a protein to form a phenyl-thiocarbamoyl derivative[152].

2) Soil chemistry

Many factors affect the decomposition of alkyl-isothiocyanates in soil. The half-life of MITC in soil has been found to vary widely[153,154,155] from 12 hours to 50 days (15°C)[155]. Morra et al. studied the decomposition of AITC in various soils[156]. The half-life of AITC in the soil ranged from 20 to 60 hours at 20°C[156]. The rate at which MITC[154,157-161] and AITC[156] decompose in soil increases with temperature. Studies have suggested that the decomposition of MITC may be hastened by microbiological degradation[153,155,158,159]. But, a recent study of AITC degradation in soil revealed no evidence of microbiological degradation[156]. Increased AITC degradation was also correlated to higher organic and nitrogen matter contents[156]. It is known that isothiocyanates can react with nucleophillic groups commonly found in organic matter[156].

Other factors in the soil environment may influence alkyl-isothiocyanate degradation. MITC is known to react with ammonia to give methyl-thiourea[162]. Application of cyanamide (H_2NCN) to MITC containing soils has been shown to increase the rate of MITC degradation[158,161]. But, the application of other ammonia based fertilizers does not significantly affect MITC degradation in soil[162]. Soil moisture strongly affects MITC diffusion and decomposition in soil[158]. The diffusion of MITC in wet soil has been shown to be irregular. In wet soil MITC was found to decompose near the point of injection into the soil[160]. AITC stability was found to increase with soil moisture over the range of 30% to 300%[156]. In another study the decomposition of MITC in soil was independent of soil moisture over the range of 8% to 20%[157].

Adsorption in soil is one of the most important aspects of a nematicide that influences its mobility and availability for biological action[158]. All previous studies of the degradation of alkyl-isothiocyanates in soil have used either carbon tetrachloride[163], ethyl acetate[153-156,161] or hexane[145] as extraction solvents. The amount of AITC extracted by hexane from a soil sample reached one-third of the initial inoculated value after 16 hours[145].

Organic solvent extractable AITC is not necessarily the form that can act as a nematicide. By using water as the extraction solvent it may be possible to estimate the free AITC available in a soil matrix to act as a nematicide.

The physical and chemical properties of AITC have been extensively studied since the late nineteenth century[164]. Interestingly, the water solubility data for this compound is unpublished. The water solubility for MITC has been determined to be 8900μg/ml[165], 8000μg/ml[166], or 7600μg/ml[6]. AITC can be expected to be less polar and therefore it should have a lower water solubility than MITC. In studies of the decomposition of AITC in aqueous solution, solutions have been prepared at concentrations of 178μg/ml[167] to 2000μg/ml[168]. Published accounts seem to suggest that AITC aqueous solubility should be between 178μg/ml[167] and less than 8900μg/ml[165] (the water solubility of MITC).

Sood and Sood studied the basic transport process in live nematodes[169]. They propose that solutes must diffuse across the outer membrane of the nematode from a surrounding water film[169]. Nematodes, in the soil, are nearly always surrounded by a water film[7]. The nematicide must have good water solubility for transport from the gas phase to the parasite[7]. The aqueous solubility of AITC is an important physical parameter that may have a strong bearing upon its nematicidal action.

3) Degradation in water

Many studies have revealed that AITC is unstable in aqueous solution[146,167,168,170]. Kawakishi and Namiki studied the decomposition of AITC in aqueous solution[168]. The proposed degradation pathway for AITC in aqueous solution is shown in **Figure 5**. All subsequent AITC decomposition studies[146,170] have assumed the degradation scheme of Kawakishi and Namiki. They determined that 14% of the AITC decomposed into elemental sulfur[168]. To further support the proposed pathway (**Figure 5**), aqueous diallyl-dithiocarbamate was observed to

degrade into diallyl-polysulfides[168]. The formation of 1,3-diallyl-thiourea (**Figure 5**) is supported by analogous reaction observed for an isocyanate (-N=C=O)[171]. The oxygen analog of an isothiocyanate is an isocyanate. In a recent study n-butyl-isocyanate was found to decompose to n-butyl-amine and 1,3-dibutyl-urea[171]. Moye et al. proposed that 1,3-dibutylurea is formed by the condensation of n-butyl-isocyanate and n-butyl-amine[171].

A series of studies have reported the decomposition of aqueous AITC but, kinetic data and the dependence of the reaction upon pH is incomplete. The half-life of AITC in an aqueous (400 µg/ml, pH=5.2) solution was 4.5 days at 37°C (calculated from reported data)[168]. Kojima and Ogawa found that AITC (1000µg/ml, distilled water) completely decomposed upon storage at 37°C for one month[146]. Leifertova et al. found that at 40°C 88% of an aqueous AITC solution degraded in 20 days[170]. A comprehensive study of the kinetics of aqueous AITC decomposition under various conditions would be of value.

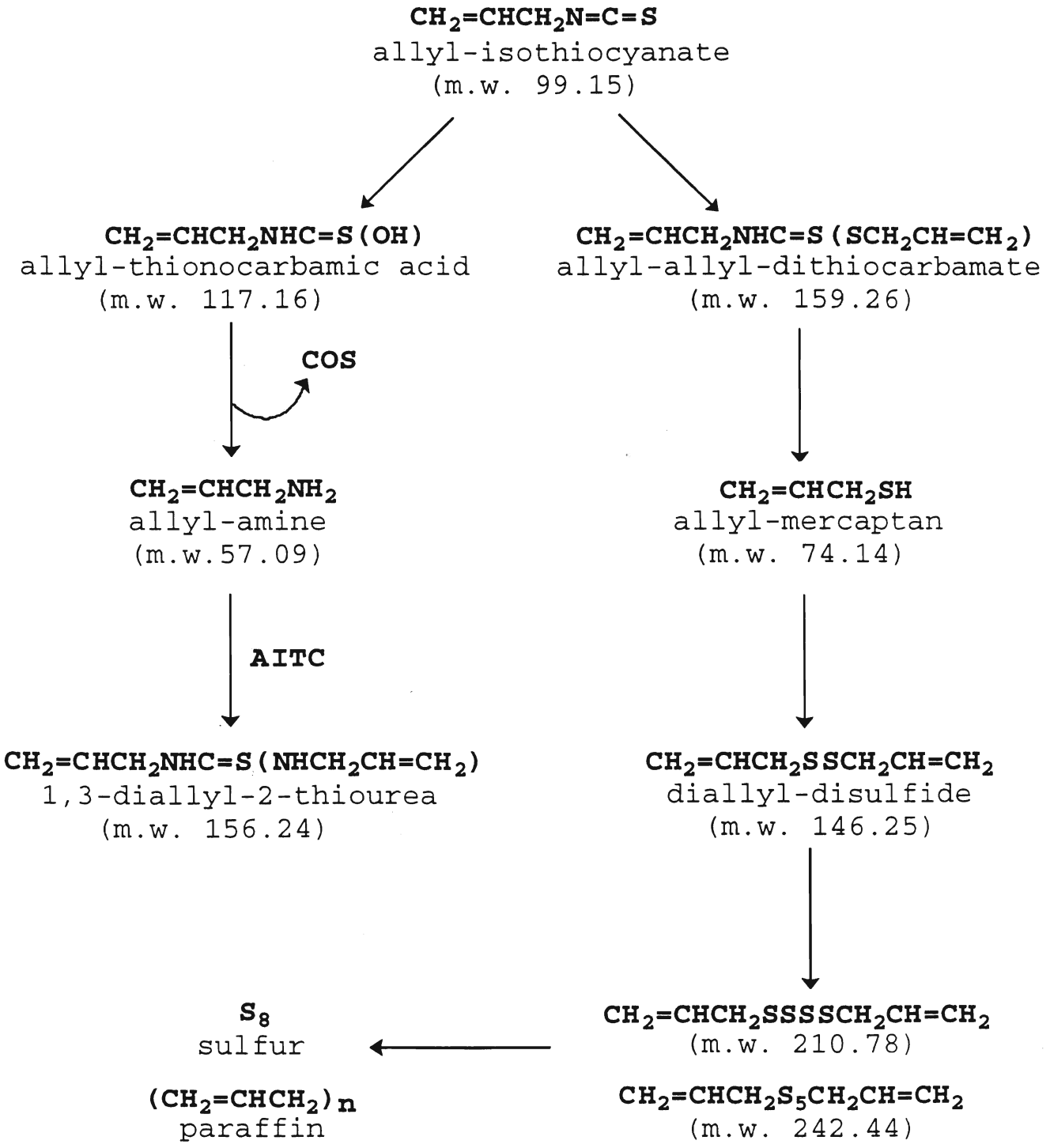
4) Methods of determination

i) Liquid chromatography

RP-HPLC methods have been developed for the determination of alkyl-isothiocyanates in aqueous solution. Mobile phases of acetonitrile/phosphate buffer[172] and 100% methanol[173,174] have been used. UV absorption detection of AITC is optimal at 200nm[174], although 254nm[173] has also been used. Kanemaru et al. developed an RP-HPLC based system for the quantification of AITC in hydrolyzed *Brassica juncea* aqueous extracts[174]. The extracts were cleaned up by SPE (C_{18}) and AITC was determined by RP-HPLC with UV absorption detection at 200nm[174]. Similarly, Terada et al. used SPE (C_{18}) for the extraction of MITC from contaminated wine[172].

In an interesting study, Hogendoorn et al. used column switching (trace enrichment) RP-HPLC for determination of MITC in aqueous solution[166]. A large sample volume (770µl) was directly

Figure 5: Degradation of allyl-isothiocyanate in aqueous solution



injected onto a trace enrichment column (C_{18})[166]. Then interfering components were eluted to waste by a low strength mobile phase[166]. The trace enrichment column was then connected to the analytical column (C_{18}), and a higher strength solvent eluted the MITC for separation, and subsequent detection[166].

Analytical methods have been developed for the simultaneous determination of MITC and sodium methyl-dithiocarbamate in aqueous samples. Mullins and Kirkbright employed ion-pair RP-HPLC for the separation[175]. Dhoot et al. used a similar method[176] except a strong anion exchange column was substituted for the RP (C_{18}) analytical column used by Mullin and Kirkbright[175]. For both methods 10mM cetyltrimethyl-ammonium bromide (CTAB) was used as the ion-pair reagent in an aqueous methanol mobile phase[175,176].

Mullin studied a series of alkyl-isothiocyanates by RP-HPLC[177]. Mullin found that isothiocyanates react with methanol[177]. Other researchers have also noticed that isothiocyanates are unstable in alcohol solution[147,167,170,178]. Alkyl-thionocarbamic acid ester ($R^1NHC(=S)-OR^2$) is the product of the reaction between an alcohol (R^2OH) and an alkyl-isothiocyanate ($R^1-N=C=S$)[147,167,170,178]. Mullin has recommended that standard alkyl-isothiocyanate solutions should not be prepared in methanol[177]. AITC has been shown to be stable in aprotic organic solvents[167,178]. To ensure compatibility between the sample solvent and the mobile phase acetonitrile should be used in place of methanol for both sample preparation and as the mobile phase organic modifier.

ii) Miscellaneous methods

Various methods for the determination and quantification of alkyl-isothiocyanates have been presented in the literature. Because of the high volatility of alkyl-isothiocyanates many analytical procedures have employed GC[179-183]. AITC is of sufficient stability so determination by GC may be accomplished. AITC was found to decompose by only 10% after a two minute column residence at 61°C[183]. GC has been used to detect alkyl-isothiocyanates

liberated from degraded *Brassica sp.* tissues[79,184-187]. Alkyl-isothiocyanates have been detected by flame ionization detection (FID)[179,185-187], flame photometric detection (FPD)[181,182] and EI-MS[79,179,183,184].

Alkyl-isothiocyanates may be characterized by mass spectrometry. EI-MS of short chain unsaturated alkyl-isothiocyanates generally give a substantial molecular cation ($[AITC]^{+\cdot}$ m/z = 99)[185,188]. For AITC fission of the carbon α to the nitrogen gives an allyl cation as the base peak of the spectrum (m/z = 41)[185,188]. The nitrogen ylid fragment at m/z = 72 is also a predominant fragment ($CH_2=N^+CS$)[185,188]. Interestingly, the mass spectra of alkyl-isothiocyanates can be distinguished from the isomeric thiocyanates. Alkyl-isothiocyanates often give a molecular ion of lower intensity than the corresponding thiocyanates[189]. Also, the sulfur ylid ($CH_2=S^+CN$, m/z = 72) fragment is practically absent from the spectra of thiocyanates.

Alkyl-isothiocyanates may be identified by infrared spectroscopy[190,191]. The asymmetric stretching mode of the isothiocyanate system causes a moderately strong band in the 2170-2221cm^{-1} region, a strong band in the 2050-2150cm^{-1} region, and a weak band at 2000cm^{-1} [190,191]. A very strong band is observed in the 1318-1347cm^{-1} region with all alkyl-isothiocyanates of the general formula $R_1-CH_2-N=C=S$. The band arises from bending vibrations of the α-methylene group[190,191].

Many other analytical techniques have been used for the determination of alkyl-isothiocyanates. For example, MITC has been trapped as the ethoxyurethane ($C_2H_5O[C_2H_4O]_2C[=S]NHCH_3$) derivative[192,193]. The derivative was quantified by RP-HPLC with UV detection at 250nm[192,193]. Isothiocyanates have also been determined by the characteristic melting point of the benzyl-thiourea derivative[194]. Verma et al. devised a method for the determination of AITC based upon the conversion into n-butyl-allyl-thiourea by the action of excess n-butyl-amine[195]. The excess n-butyl-amine was determined by potentiometric titration[195]. In a separate study a

colorimeteric determination of AITC has been developed based upon the formation of methylene blue and UV absorption at 665nm[196]. Alkyl-isothiocyanate mixtures have been separated by thin layer chromatography as the thiourea derivative or in the intact form[197].

6. Scope of the study

The general goal of this thesis was to study parameters related to the development of *Brassica juncea* as a sustainable nematicide. *Brassica juncea* was selected because various studies have shown that the plant is characterized, almost exclusively, by the glucosinolate sinigrin[23,42,43]. Sinigrin is the precursor to the nematicidal agent AITC[50,51]. AITC can be expected to be a very active nematicidal agent[27,32].

A goal of the present study was to determine the distribution of the glucosinolate sinigrin in *Brassica juncea* (cvs. Cutlass and Domo). A comparison was made of the sinigrin levels in various tissues. Sampling was performed throughout the growing season. Among the necessary experiments was the development of a simple and reliable method of extraction of sinigrin from *Brassica juncea* tissues.

Studies of glucosinolate extraction from *Brassica sp.* tissues have been reported in the literature (see **Table 1**). From the literature it is not clear if water or aqueous methanol is a better extraction solvent. In many cases water has been shown to be an effective extraction solvent for *Brassica sp.* seed meals (see **Table 1**). Aqueous methanol has been employed for the extraction of glucosinolates from *Brassica juncea* green tissues[43,77] although glucosinolates are less soluble in methanol than in water[76].

Studies have shown the utility of diffusion extraction for the extraction of glucosinolates from whole *Brassica sp.* seeds[80,85]. Diffusion extraction has also been applied to fleshy tissues such as cotyledons[77]. No rigorous study exists in the literature regarding the diffusion extraction of sinigrin from *Brassica juncea*

fleshy tissues. For this report a unique study was initiated to determine the optimal time necessary for the diffusion extraction of sinigrin from whole *Brassica juncea* leaves. Both water and aqueous methanol were tested as extraction solvents.

For this investigation, various approaches were applied to the determination of sinigrin. RP-HPLC-UV based procedures were developed employing both ion-pair reagents and buffer salts in the mobile phase. The first reported NCI-MS study of glucosinolates is presented in this thesis. The PB HPLC-MS interface was used for sample introduction.

To discover critical parameters related to the use of AITC as a sustainable nematicide, selected physical properties of AITC were studied. In particular, the solubility of AITC in water was determined. AITC is unstable in aqueous solution[146,167,168,170]. The half-life of AITC degradation in aqueous solution was determined. A RP-HPLC-UV based procedure was developed to separate AITC from its aqueous degradation compounds. The pathway of AITC degradation in aqueous solution, has been reported (**Figure 5**)[168]. The contribution of each branch of the degradation pathway was studied. Also, additional aqueous AITC degradation products were discovered.

AITC degradation in soil was compared to the degradation in aqueous solution. The initial adsorption of AITC in the soil medium was estimated. Finally, in a collaborative effort with Dr. Potter's laboratory, the EC_{50} of aqueous AITC against root-lesion nematodes (*Pratylenchus penetrans*) was determined.

EXPERIMENTAL

1. Instrumentation

1) HPLC apparatus

The high performance liquid chromatography (HPLC) instrument used was the Hewlett Packard (HP) 1090 liquid chromatograph. The HP 1090 HPLC system was equipped with a 79835A solvent delivery system, a 79846A autoinjector, and a 79847A autosampler. Peak detection was performed by a 1040A diode array ultraviolet (UV) absorption detector. The HPLC system was controlled by HP 1090 software on an 85B HP computer. Integration, analysis and plotting were also under the guidance of the 85B computer.

Reversed phase (C_{18}) analytical columns and precolumns were employed for the analysis of samples. A Supelco 20 x 4.6 mm pelliguard C_{18} precolumn and a Supelco 0.45 micron frit were placed in line before the analytical column.

2) HPLC particle beam mass spectrometer

The mass spectrometer used was the Kratos Concept IS double-focusing E/B configuration type machine. The source was operated in CI mode at a temperature of 180°C with 70eV potential. A scan rate of three seconds per decade for full scan acquisition was used. A gain of 4.5 to 5.0 was used which gave a noise level of $6x10^3$ to $1x10^4$ counts. The instrument was set with a nominal resolving power of $1x10^3$. Perfluorokerosene (PFK) was used as a calibration standard. The mass spectrometer was interfaced to a Kratos DART speed data acquisition and control system linked to software running on a Sparcstation 10 computer. This system was used for all data management and processing.

The sample was introduced to the particle beam interface by a Waters 600-MS pump system operating with a flow rate of 500µl/minute. Sample injections were made into the mobile phase

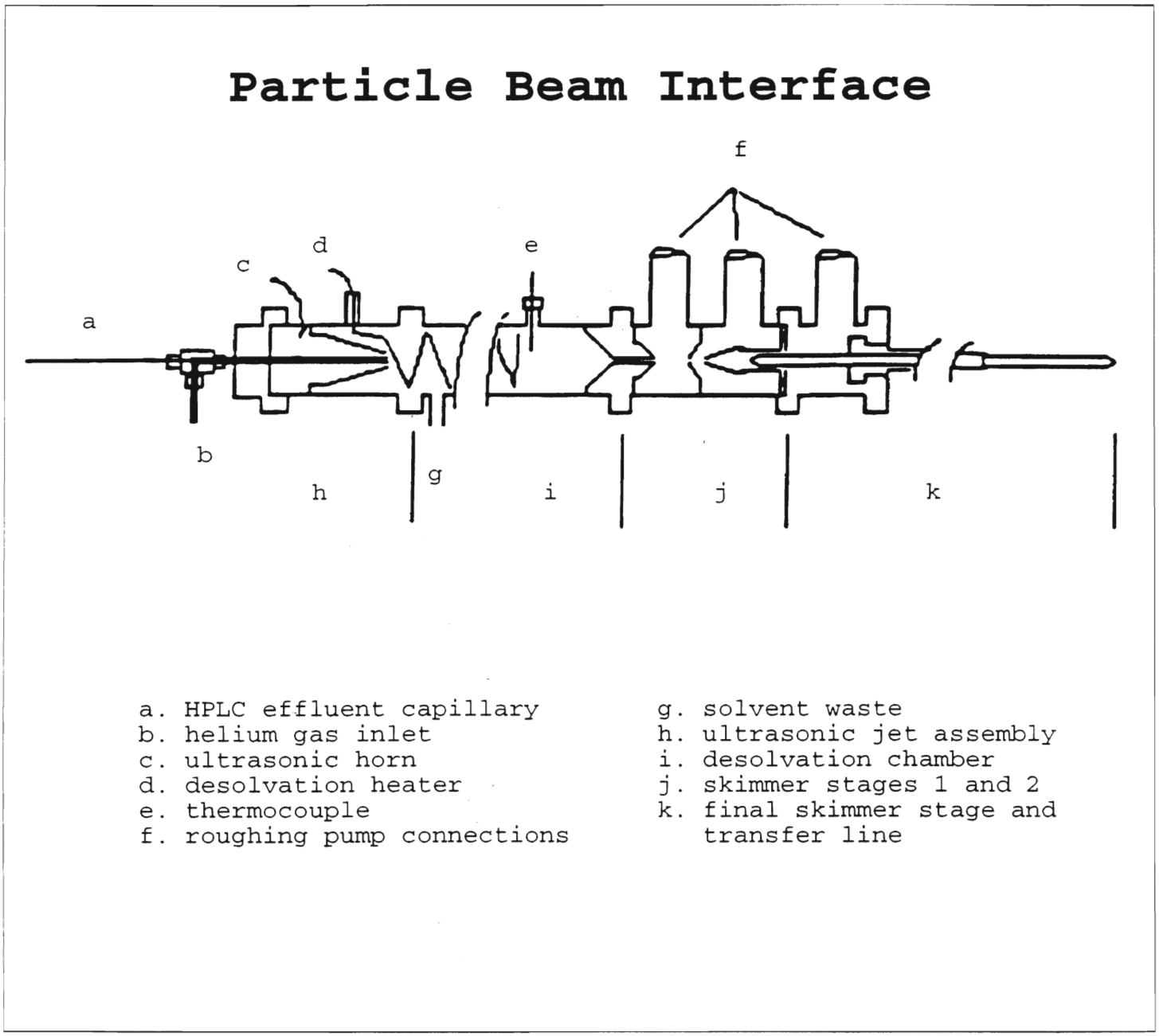
stream through a Rheodyne model 7000 injection valve with a 20µl sample loop. A mobile phase solution of 100% methanol was used.

The particle beam (PB) HPLC-MS interface designed by Singh et al.[133] was used for the particle beam negative chemical ionization mass spectrometry (PB-NCI-MS) study of glucosinolates. The schematic diagram of the particle beam interface is shown in **Figure 6.** The effluent from the HPLC was delivered by a deactivated silica capillary (0.075mm i.d., 0.34mm o.d.) to the ultrasonic horn of the particle beam interface. An ultrasonic transducer was in the body of the ultrasonic horn. The effluent was nebulized at the exit end of the ultrasonic head by a concentric flow of helium gas at 900 ml/minute. The first skimmer stage of solvent stripping involved a Welch Duo Seal vacuum pump (500 L/minute pump speed) connected to two diametrically opposed pumping ports. Ports of the second identical stage were connected to an Edwards high vacuum pump with 300 L/minute pump speed. The final skimming stage was pumped by an Edwards high-vacuum pump with 150 L/minute pump speed. Then the particle beam was introduced into the mass spectrometer by the transfer line. Methanol was used as the NCI reagent gas. The vacuum pumps on the skimming stage were adjusted to give a source methanol pressure of 10×10^{-5} Torr.

3) General

Measurements of pH were made with a Horiba F-13 pH meter calibrated with a three-point calibration. The calibration buffer solutions were supplied by Horiba: pH=10.011 $\pm$ 0.005, 7.000 $\pm$ 0.002 and 4.004 $\pm$ 0.002 (25°C). Standard buffer solutions were supplied by BDH to allow for the accurate pH measurement outside the three-point calibration range; pH=11.0 (lot 903081) and 12.0 (lot 903082).

Figure 6: Schematic Diagram of the Particle Beam Mass Spectrometer interface



2. Materials

1) Chemicals and solvents

The mobile phase solutions used in this study were organic solvents, reverse osmosis water, buffer solutions and aqueous ion pair reagent solutions. Generally, the mobile phase solutions were filtered and vacuum degassed through a 0.45 micron Teflon filter (Pall) before use. Mobile phase solutions were sparged by a constant stream of helium gas.

HPLC grade acetonitrile and methanol were supplied by Caledon Laboratories Ltd. Reverse osmosis water was used in the preparation of all aqueous solutions. The reverse osmosis water was prepared at Agriculture and Agri-Food Canada, Pest Management Research Centre, Vineland Station, Ontario. In this report water refers to reverse osmosis water.

Aqueous cetyltrimethyl-ammonium bromide (CTAB) solutions were employed as both an ion pair reagent mobile phase solution additive and a solid phase extraction elution solvent. CTAB was supplied by Caledon (98%, lot 07502). Aqueous CTAB solution (1mM) was prepared from approximately 0.38g of CTAB in 1L water. Aqueous 0.1M CTAB was prepared from approximately 0.93g of CTAB dissolved in 25ml water. The solubility of CTAB in water at 26°C is approximately 0.1M. Dissolution or crystallization occured slowly; slight warming to 28°C rapidly dissolved the precipitate. CTAB solutions used as an ion pair mobile phase solution were not vacuum degassed/filtered or sparged. Filtering or sparging of CTAB solution caused excessive foam formation.

Additional reagents and solvents used are listed below:

- Glacial acetic acid (CH_3CO_2H) was supplied by Baker (analyzed reagent, 99.9%)

- Acetone was supplied by Caledon Laboratories (HPLC grade).

- Ammonium acetate ($CH_3CO_2^-NH_4^+$) was supplied by Fisher Scientific (ACS grade, lot 705370).

- Allyl-amine was supplied by Aldrich Chemical Company (98%, lot 04401TZ).

- Technical grade allyl-mercaptan was supplied by Aldrich Chemical Company (80%, lot EG11405DG).

- Carbon disulfide was supplied by Matheson, Coleman and Bell (Spectroscopic grade [>99%], lot 8J23A).

- Diethyl ether was supplied by Anachemia (distilled in glass, stabilized with 2% ethanol).

- Anhydrous disodium hydrogen phosphate (Na_2HPO_4) was supplied by Fisher Scientific (99.9%, lot 734815).

- Potassium dihydrogen phosphate (KH_2PO_4) was supplied by Fisher Scientific (HPLC grade, 100.1%, lot 880603).

- Sodium metal was supplied by BDH Chemical Company (reagent grade, metal stick in liquid paraffin).

- Sodium hydroxide (NaOH) was supplied by Fisher Scientific (pellets, certified ACS grade, 98.4%, lot 864495).

- Thioglucosidase (myrosinase) was supplied by Sigma Chemical Company (lot 11H0048, 290units/g, one unit gives 1.0mmol glucose/minute at pH=6.0 and 25°C). The crystalline solid was stored at -17°C.

2) Buffer solutions

Phosphate buffer solutions were prepared from Na_2HPO_4 and KH_2PO_4 stock solutions. Buffer solutions of pH=5.3 to 8.0 were prepared

from mixtures of the stock phosphate solutions. A 0.07M Na_2HPO_4 stock solution was prepared from approximately 37.9g Na_2HPO_4 dissolved in 4L water. It should be noted that Na_2HPO_4 should be added slowly to water with stirring or else a hard, and difficult to dissolve, precipitate will form. A 0.07M KH_2PO_4 stock solution was prepared from approximately 36.3g of KH_2PO_4 dissolved in 4L water. Both stock phosphate solutions were filtered through 0.45 micron Teflon filters (Pall). The stock solutions were stored in the dark at 1°C to prevent micro-organism contamination.

As an example, 0.7mM, pH~6.8 buffer solution was prepared from 5ml 0.07M Na_2HPO_4 and 5ml 0.07M KH_2PO_4. The mixture was diluted with water to 1L in a volumetric flask. This buffer was used as a mobile phase solution and for the extraction of sinigrin from *Brassica juncea* tissues. Buffer solutions were prepared daily to avoid microorganism contamination.

Aqueous ammonium acetate (0.1M) buffer solution was prepared from approximately 7.7g of $CH_3CO_2^-NH_4^+$ dissolved in 1L of water. The pH of the aqueous $CH_3CO_2^-NH_4^+$ solution was approximately 6.4. The ammonium acetate buffer solution was prepared fresh daily, and vacuum degassed through a 0.45 micron Teflon filter (Pall) immediately before use.

The following series of buffers were prepared (as media for the degradation study of allyl-isothiocyanate in water): pH=11.69 with Na_2HPO_4/NaOH, pH=6.38 with KH_2PO_4/NaOH, and pH=3.91 with CH_3CO_2H/NaOH.

Na_2HPO_4/NaOH buffer solution, pH=11.69, was prepared from 500ml of the above Na_2HPO_4 stock solution and 270ml of approximately 0.1N NaOH diluted (after mixing) to 4L with water. Sodium hydroxide solution was prepared immediately before use from 4.23g of NaOH pellets dissolved in 1L of water.

KH_2PO_4/NaOH buffer solution, pH=6.38, was prepared from 500ml of the stock KH_2PO_4 solution and 56ml of approximately 0.1N NaOH

solution diluted to 4L with water.

The CH_3CO_2H/NaOH buffer solution, pH=3.91, was prepared from 500ml of 0.1N CH_3CO_2H solution and 50ml of 0.1N NaOH solution diluted (after mixing) to 4L with water. The 0.1N CH_3CO_2H was prepared from 5.7ml of glacial acetic acid diluted to 1L with water.

3) Standard solutions

Standard solutions prepared from the following chemicals were stored in the dark between -4°C and 1°C. The bulk chemicals were stored in the dark at -4°C.

- Allyl-isothiocyanate (AITC) was supplied from the Aldrich Chemical Company (95%, lot 00531KZ), which was supplied as a colorless pungent oily liquid. AITC stock solutions for HPLC analysis were prepared in 100% acetonitrile (2287, 11.43, 45.69, 114.3, 52.61, 228.6, 571.2, and 1143µg/ml).

- Allyl-thiourea was supplied from Aldrich (98%, lot MF09230HF) as a white crystalline powder. Allyl-thiourea stock solutions for HPLC analysis were prepared in water (1193µg/ml).

- The potassium salt of benzyl-glucosinolate was supplied by Merck Chemical Company (>99%, lot 837YW160458). The solid sample was stored at -17°C. Benzyl-glucosinolate stock solutions for HPLC analysis were prepared in water. Samples for mass spectrometry were prepared in 100% methanol (1000µg/ml).

- 1,3-Diallyl-2-thiourea (DATU) was supplied by the Aldrich Chemical Company from the Sigma-Aldrich library of rare chemicals (purity unstated, no lot given). DATU was supplied as a slightly yellowish white crystalline solid. DATU stock solutions for HPLC analysis were prepared in 40%

acetonitrile/water (24.90 and 113.5µg/ml).

- Diallyl-sulfide was supplied by the Aldrich Chemical Company in the technical grade (80%, lot TF15613TF). Diallyl-sulfide stock solutions for HPLC analysis were prepared in 100% acetonitrile (16.96, 33.92 and 169.6µg/ml).

- Diallyl-disulfide was supplied by Sigma (lot 54H2621). Diallyl-disulfide stock solutions for HPLC analysis were prepared in 100% acetonitrile (241.0µg/ml).

- The potassium salt of sinigrin (monohydrate) was supplied by Sigma (purity unstated, lot 70H7090). The solid sample was stored in the freezer at -17°C. Sinigrin stock solutions for HPLC analysis were prepared in water (4.707, 10.46, 52.31, 104.6 and 1046µg/ml). Sample solutions used for mass spectrometry were prepared in 100% methanol (998.0µg/ml).

3. Procedures

1) General

Unless otherwise stated all solutions were prepared in volumetric glassware. Liquid-liquid mixtures are expressed as a percentage volume of each component. All volumetric glassware and pipettes were cleaned with RBS-35 (dichromate-sulfuric acid substitute) detergent solution (Pierce) or soap and water. Occasionally, glassware was cleaned by soaking overnight in 30% nitric acid/water. After cleaning, the glassware was rinsed with water and oven dried.

2) Plant propagation

Both Cutlass and Domo (*Brassica juncea*) cultivars (cv.) were grown under artificial lights in a controlled plant growth chamber. Balanced artificial sunlight was achieved by using both sodium and

mercury vapor lamps. The light source was fixed at approximately 120cm from the soil surface. The intensity of the light was measured at soil level with a Sterling LX-101A (K99635) lux meter. The mean value and standard deviation for a series of fifteen readings in the growth room was 8940 $\pm$ 1800 lux. Temperature was maintained between 17.5°C and 21.5°C. Relative humidity was set at approximately 60%. The length of the day was set to 14 hours.

Additional *Brassica juncea* (cv. Cutlass) plants were grown in a smaller growth chamber (Conviron CMP 3244) to obtain leaf samples to develop a sinigrin extraction method. The balanced light source was a combination of incandescent and fluorescent lamps. The length of the day was set at 14 hours. Average light intensity was determined to be approximately 9900 lux. The approximate temperature and relative humidity of the chamber was measured as 21.5°C and 61.8%, respectively. An Hanna Instruments HI therm-hygrometer was used for temperature and humidity measurements.

The plants were grown from seed. Two seeds were planted in a growth pot approximately 4 by 10cm. The growth pot contained a standard, sterilized, greenhouse soil medium. The soil medium was prepared at Agriculture and Agri-Food Canada (Vineland Station). Plants were watered daily and fertilized once per week with a standard, balanced, liquid plant food.

The seedling pots were randomly distributed in the light area. The positions of the pots were randomly changed weekly to reduce local light deviations. Laggard or bolting plants were discarded. A crop uniform in both plant height and leaf number was maintained for the extraction experiments.

3) Plant sample preparation

Generally, plants were harvested weekly. The first three and fully mature true leaves (or specific tissues) were sampled. During the first two weeks the whole seedling was sampled. The seedlings were cut at the soil level. Only fully green material was selected.

Wilted, damaged or mildew contaminated material was discarded. Care was taken to randomly select the plant material. Plant material was harvested when the exterior of the plant was dry. Soil particles were wiped from the leaf surfaces with a dry tissue paper (Kimwipes). Care was taken to avoid bruising the plant material during harvest. After harvesting and recording the fresh tissue weight, the extraction procedure was quickly started.

4) HPLC operation

Separations were done with the HPLC analytical column compartment at ambient temperature (approximately 27°C). The standard injection size used was 25µl. Analytical methods were calibrated by external standards of the compounds of interest.

Before HPLC analysis, all samples were filtered through 1.0 micron Acrodisc CR syringe filters (Gelman Sciences). The syringe filter membrane was made of Teflon (PTFE). The syringe filters were certified to be resistant to acetonitrile and methanol. After filtering the samples were stored in glass vials.

4. Experiments

1) Development of methods

i) Extraction of sinigrin from *Brassica juncea*

Leaves were selected randomly from approximately 500 flowering plants. The plants were grown as previously described and were 5 weeks old at the time of harvest. The plants at harvest were approximately 35cm high. Only dark green, fully mature, true leaves were selected. The aqueous buffer and 70% methanol extractions were completed with plant material from the same set of plant pots. Both extractions were performed on the same day.

a) Aqueous buffer solution

The procedure given below was used to determine the optimal time of extraction of sinigrin from *Brassica juncea* (by aqueous phosphate buffer solution). A 100ml round bottom flask was fitted with a reflux condenser and a rubber septa stoppered side arm. The reflux condenser was open to the atmosphere. The flask was heated by a heating mantel. Aqueous phosphate buffer solution (800ml, pH=6.38) was brought to reflux in the flask. A sample of *Brassica juncea* cv. Cutlass leaves (25.25g, sampling previously described) were added to the solvent and a stopwatch was started. Every 5 minutes 1.5ml aliquots of the refluxing mixture were withdrawn via a 2ml syringe. The samples were filtered and immediately stored in a Teflon sealed sample vials for later HPLC analysis.

The extraction procedure below was developed for the determination of weekly concentrations of sinigrin in *Brassica juncea* tissues. Sinigrin was extracted from the *Brassica* tissue by an aqueous phosphate buffer solution (0.7mM, pH~7). Buffer solution (40ml) was added to a flask equipped with a reflux condenser. The buffer solution was brought to a boil. Approximately 5.0g of plant material (sampling previously described) was plunged into the boiling buffer. Immediately an additional 30ml of boiling buffer was added to the flask. The time of addition of the plant material was noted. After 25 minutes the heat source was removed and the condenser was washed down with 10ml of buffer.

The extract was decanted through a small glasswool plug into a 100ml volumetric flask. The extraction flask and glasswool were washed with 10ml of buffer. Residual extract was pressed out of the glasswool plug with the bottom of a 10ml beaker. One millilitre of 0.1M CTAB solution was added to the extract. After the extract had cooled to room temperature the fluid level in the volumetric flask was adjusted to the calibration mark with buffer to give 100ml of extract. The final concentration of CTAB in the extract was 1mM.

b) Methanol/water (70:30 v/v)

An optimal time of extraction study with 70% methanol/water was accomplished in a manner similar to the method above (aqueous phosphate buffer). The solvent employed was 800ml of 70% methanol/water. The amount of plant material used was 29.69g.

ii) *Brassica juncea* extract clean up

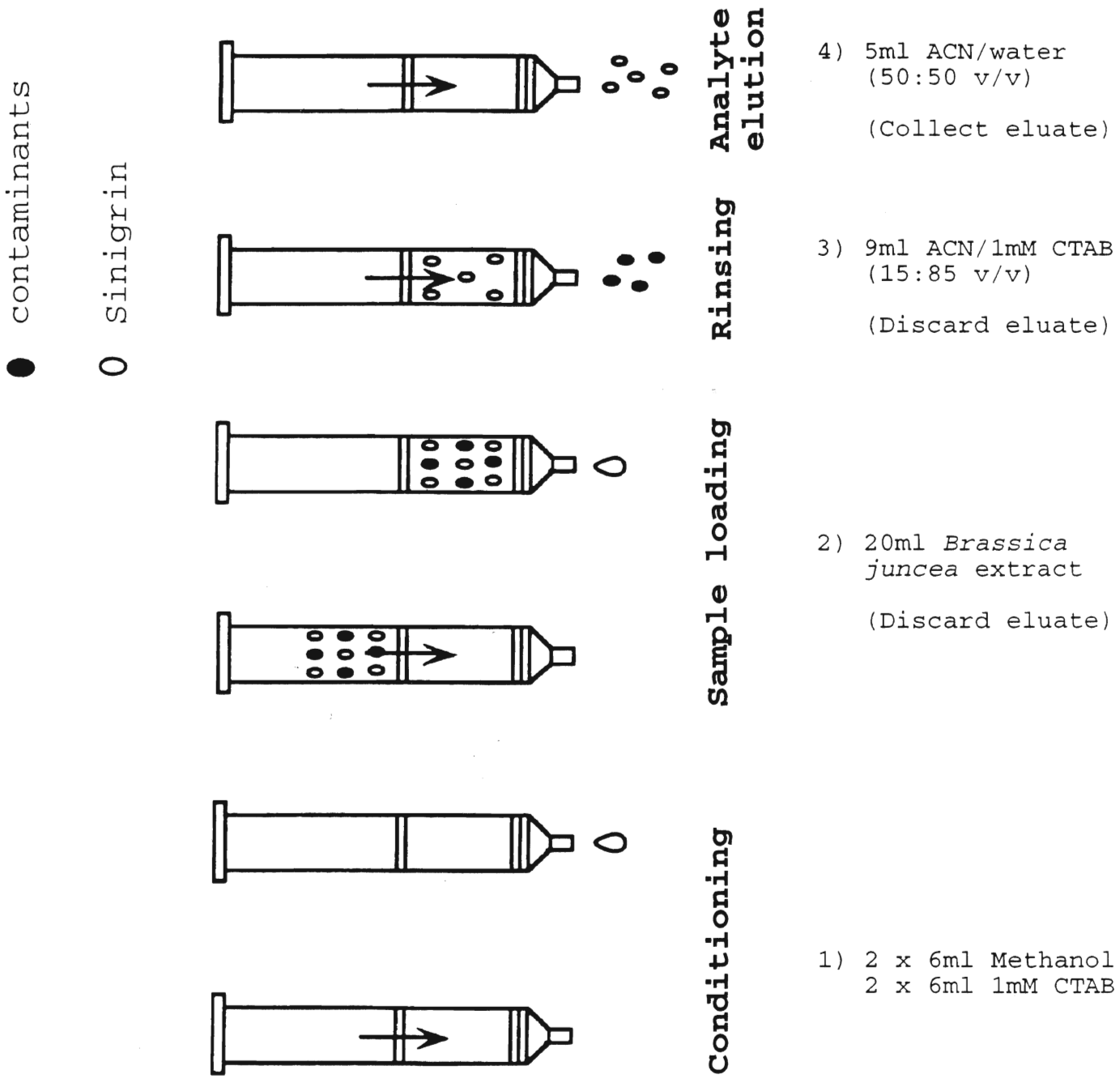
The aqueous phosphate buffer extracts of weekly harvested *Brassica juncea* were cleaned up by solid phase extraction. The solid phase adsorbent employed was Spe-ed C_{18} (carbon load 14%) chemically bonded silica (Applied Separations, lot 952079). The solid phase adsorbent was supplied as 500mg in 6ml cartridges. The Spe-ed mate 30 vacuum manifold was used to draw sample and eluting solvents through the cartridges. On the manifold a vacuum was maintained between -60 and -50KPa. The flow through the cartridge was adjusted to 6ml per 20 seconds. Flow was stopped when the sample was approximately 2mm above the solid phase bed. Eluates were collected in 25ml volumetric flasks.

The SPE clean up procedure employed (**Figure 7**) was as follows. The cartridge was conditioned by washing twice with 6ml methanol. Then the cartridges were washed twice with 6ml of 1mM aqueous CTAB solution. The sample (20ml) was then loaded on the cartridge. The cartridge was washed with 9ml of 15% acetonitrile/1mM CTAB solution. Then 5ml of 50% acetonitrile/water solution was drawn through the cartridge to remove the retained sinigrin. The final fraction was collected in a 25ml volumetric flask. The volume was adjusted to the calibration mark with water. The concentration of acetonitrile in the final fraction was 10%. SPE cartridges were discarded after a single use.

iii) Stability of sinigrin in boiling buffer solution

A 250ml round bottom flask was fitted with a reflux condenser and a rubber septa stoppered side arm. The flask was heated with an

Figure 7: Ion pair solid phase extraction clean up of *Brassica juncea* extracts



electric heating mantel and stirred with a magnetic stir bar (Teflon coated). Initially, 180ml of phosphate buffer solution (pH=6.76) was brought to reflux in the flask. Sinigrin, 20ml (approximately 1000µg/ml, in pH=6.76 phosphate buffer solution), was added to the refluxing solution. The time was noted at the instant of addition. Every five minutes a 1.5ml aliquot was taken from the refluxing mixture by a 2ml syringe. The sample was immediately stored in a sealed glass vial for later HPLC analysis.

iv) Development of RP-HPLC-UV methods

a) Sinigrin determination: phosphate buffer mobile phase

The sinigrin concentration of the aliquots collected from the sinigrin stability study was determined by an RP-HPLC-UV method employing an isocratic aqueous phosphate buffer solution (pH~7). The basic HPLC instrumentation previously described was used. Mobile phase solution flow rate was maintained at 1ml/minute. The analytical column used was a Phenomenex Ultracarb 5 micron ODS (30% carbon load) 150 x 4.6 mm (# PP/4352C). Sinigrin peak area was monitored at 228nm. Total analysis time was approximately 3 minutes.

b) Sinigrin determination: acetate buffer mobile phase

Aqueous phosphate buffer *Brassica juncea* extracts (from the optimal time of extraction study) were analyzed for sinigrin concentration by an RP-HPLC-UV method employing an ammonium acetate mobile phase solution. The basic HPLC instrumentation previously described was used. A CSC-5 ODS-2, 5 micron packing, 250 x 4.6mm (CSC, #069293) column was employed as the analytical column. The isocratic mobile phase solution consisted of 0.1M aqueous ammonium acetate run at a flow rate of 1ml/minute. The total analysis time was approximately 6 minutes. Sinigrin peak area was monitored at 228nm.

c) Sinigrin determination: ion pair mobile phase

Brassica juncea extracts (70% methanol, from the optimal time of extraction study) were analyzed for Singrin concentration by an RP-HPLC-UV method employing an ion pair reagent in the mobile phase solution. The basic HPLC instrumentation previously described was used. An HP ODS 5 micron Hypersil 100 x 2.1mm analytical column (Hewlett Packard, #799160D-552) was employed. An isocratic mobile phase solution of 22% acetonitrile/63% 0.1M ammonium acetate/15% 1mM CTAB was used. The flow rate of the mobile phase solution was 700µl per minute. The total analysis time was approximately 6 minutes. Sinigrin peak area was monitored at 228nm.

d) Sinigrin determination: step elution method

A method employing a step gradient was developed for the determination of sinigrin in aqueous buffer *Brassica juncea* extracts. This method was used for the determination of sinigrin concentrations in weekly harvested *Brassica juncea* samples (Cutlass and Domo). Before analysis, the *Brassica* extracts were cleaned up by the SPE method previously described. The analysis was accomplished by using the standard HPLC instrumentation. The mobile phase solution program consisted of a preanalysis conditioning stage involving a phosphate buffer solution (0.05M, pH~7) for five minutes at 1.0ml/minute. At the time of injection the mobile phase solution switched to 100% water. The flow rate of the mobile phase solution was maintained at 1.0ml/minute.

A large series of *Brassica juncea* extracts were analyzed by the above method. Two different analytical columns were used during the analysis series: CSC-5 ODS-2, 5 micron packing, 250 x 4.6mm (CSC, #069293), and Vydac C-18 250 x 4.6mm (#157 900706). The 1040A diode array detector was set to monitor sinigrin peak area at 228nm.

e) Determination of AITC and aqueous degradation compounds

An analytical method was developed to separate and quantify AITC in the presence of its aqueous degradation products. The analysis was performed with the basic HPLC instrumentation. The following gradient mobile phase system was used; the system was started with 100% water which was linearly changed to 43% acetonitrile/water in 19 minutes then the mobile phase was linearly increased to 100% acetonitrile in 7 minutes and held at 100% acetonitrile for 5 minutes. The mobile phase solution flow rate was maintained at 1.0ml/minute. The total analysis time was 31 minutes with a five minute post-analysis time at 100% water to bring the system back to starting equilibrium.

A reversed phase CSC-5 ODS-2, 5 micron packing, 250 x 4.6mm analytical column was employed for the separation (CSC, #069293). The 1040A diode array detector was set to monitor the absorption at 200nm, 228nm, 240nm and 260nm simultaneously. Furthermore, the UV absorption spectra of each observed peak were acquired during the analysis. The method was regularly calibrated by external standards of allyl-isothiocyanate and 1,3-diallyl-2-thiourea.

v) Determination of sinigrin and benzyl-glucosinolate by HPLC-PB-NCI-MS

The negative chemical ionization mass spectrometry (NCI-MS) of the glucosinolates sinigrin and benzyl-glucosinolate were studied. The samples were introduced into the mass spectrometer by the particle beam interface previously described (**Figure 6**). Sinigrin and benzyl-glucosinolate standards were prepared in 100% methanol and injected directly into the HPLC effluent stream. The mass spectrometer was operated in the full scan mode under NCI conditions as previously described. The mass spectrometer was equilibrated for approximately 1.0 to 1.5 hours before spectra were acquired.

2) Determination of sinigrin in *Brassica juncea*

Studies were conducted to determine the concentration of sinigrin in the top three leaves of *Brassica juncea* cultivars Cutlass and Domo. Also, the sinigrin concentration of other plant parts was determined at the fifteenth week after planting. The methods of plant propagation and sampling were previously described. Extraction of the plant tissue was accomplished with boiling phosphate buffer solution, pH~7 (0.7mM), for 25 minutes as previously described. The extracts were cleaned up by SPE and the sinigrin concentration was determined by the step gradient RP-HPLC-UV method previously described.

3) Behaviour of AITC

i) Solubility of AITC in water

Aqueous solutions of AITC with concentrations between 100 and 1800µg/ml were made. In 25ml volumetric flasks the desired amount of AITC was added via a micro syringe. 10ml of water was added and the resulting suspension was shaken for one hour. The solutions were allowed to sit undisturbed and were used within twelve hours.

A Varian DMS-100 UV-Vis double-beam spectrophotometer was employed to determine the concentration of dissolved AITC in the prepared solutions. A portion of the supernatant layer was removed with a pipette. Within a quartz sample cuvette (1cm) the homogeneous supernatant was diluted to one sixth of the original concentration with water. The absorbance of the resulting solution was monitored at approximately 240nm. The peak abosrbance value in this region was noted. For each solution, after measuring the absorbance, the cuvette was rinsed successively with water, methanol, RBS cleaning solution (with methanol added to 20% v/v), and finally with water. One sixth sample dilution was selected to avoid saturation of the detector.

ii) Degradation of AITC in water

Aqueous AITC solutions were prepared with a buffer solution of pH=6.30 (335 and 1084µg/ml). The desired amount of AITC was added to a volumetric flask. The volumetric flask was half filled with the buffer solution and shaken for approximately one hour. Then the flask was filled to the calibration mark with the buffer solution and covered with aluminum foil (to exclude light). The flasks were then sealed with ground glass stoppers and wax film (Parafilm). Solutions were stored at room temperature (approximately 25°C).

Aqueous AITC samples were prepared to study the degradation of AITC at 35°C. Solutions were prepared with buffer solutions of pH=4.97 to 9.07. The AITC concentration was approximately 1000µg/ml. The aqueous samples were prepared as described above.

Samples were prepared for HPLC analysis by thoroughly shaking the solution. One millilitre of the sample was drawn up in a 2ml syringe then an additional 1ml of 100% acetonitrile was drawn up in the same syringe. The mixture was transferred into a glass HPLC vial and tightly covered for subsequent HPLC analysis.

iii) Degradation of AITC in soil

The degradation of AITC in soil at 35°C was studied. The soil was acquired from Alliston, Ontario. The soil was sifted through a 0.5mm screen before use. Soil moisture content was 12.65±0.06%. Approximately 10g of soil was loaded into 25ml scintillation vials. The soil was inoculated with 1ml (via a pipette) of 1005.85µg/ml AITC in pH=6.52 phosphate buffer solution. Then, the soil/AITC mixture was stored at 35°C. Approximately every 20 hours the soil samples were extracted by adding 9ml of water. The vial was well shaken and the solids in the slurry were allowed to settle for 10 minutes. The solution was taken up in a 1ml syringe and filtered through a 1.0 micron Acrodisc CR syringe filter (Gelman Sciences). AITC concentration in the filtered sample was determined by the RP-HPLC-UV method previously described.

4) Synthesis of AITC degradation compounds

i) Allyl-allyl-dithiocarbamate

$CH_2=CHCH_2-NCS + CH_2=CHCH_2S^- Na^+ \rightarrow CH_2=CHCH_2-NHC=S(-SCH_2CH=CH_2)$

A 250ml round bottom flask was fitted with a reflux condenser and a rubber septa stoppered side arm. Allyl-mercaptan (14.37g) was stirred in the flask and the flask was placed on an ice bath. To the allyl-mercaptan was added 0.60g of sodium metal. The sodium metal was washed with methanol just before use to expose the active metal and to remove the paraffin oil. The sodium metal was cut into small pieces to speed the reaction. AITC (5.19g) was then added to the sodium allyl-mercaptide solution. AITC was added over 5 minutes and the solution was stirred over night. The resulting solution containing allyl-allyl-dithiocarbamate was yellow in color.

Allyl-allyl-dithiocarbamate was isolated from the reaction mixture by an RP-HPLC separation method. The standard HPLC instrumentation was used. A CSC-5 ODS-2, 5 micron packing, 250 x 4.6mm (CSC, #069293) column was employed as the analytical column. The isocratic mobile phase solution consisted of 30% acetonitrile/water. A portion of the reaction mixture (100µl) was diluted with 1ml of acetonitrile. The diluted reaction mixture was injected (25µl) and the fraction eluting between 34 and 37 minutes was collected. The total time of the separation method was 90 minutes. Injections were repeated until approximately 50ml of the fraction was collected.

ii) Sodium allyl-dithiocarbamate

$CS_2 + NaOH + CH_2=CHCH_2-NH_2 \rightarrow CH_2=CHCH_2-NHCS_2^- Na^+$

A 250ml round bottom flask was fitted with a reflux condenser and a rubber septa stoppered sidearm. In the flask 11ml of carbon disulfide and a cold solution of 7.22g of sodium hydroxide

(pellets) in 16ml of water was stirred.

Over one hour, 40ml of a solution of allyl-amine in water (30% v/v) was added. Initially, addition of the amine solution produced a yellow solution with a white precipitate. After three hours of stirring the solution was homogeneous and bright orange. The solution was stirred overnight before workup.

The solvent was removed under vacuum. The resulting orange solid was recrystallized from acetone/diethyl ether (50:50 v/v) 400ml. 3.96g of an cream-white solid was obtained after two recrystallizations from acetone/diethyl ether and washing with 15ml portions of diethyl ether. Mother liquors were allowed to stand for two days upon which 2.44g of a pure white crystalline solid was collected. This solid was washed twice with 15ml diethyl ether.

5) Toxicological aspects of AITC

The toxicity of aqueous AITC to root-lesion nematodes (*Pratylenchus penetrans*) was determined. Aqueous AITC solutions were prepared in water and analyzed as previously described. One millilitre of a stock nematode solution (approximately 100 nematodes/ml) was added to 99ml of aqueous AITC solution. The nematode/AITC mixture was stoppered and placed on a shaker (100rpm) for one hour. The mixture was then filtered through a wet 0.5mm mesh screen. The flask was rinsed with water and the liquid was passed through the 0.5mm mesh screen. The mesh screen was backflushed into a test tube. The test tube was refrigerated overnight. Then, the number of live and dead nematodes in the test tube sample were counted.

A *Brassica juncea* (cv. Domo) flower extract was treated with myrosinase to produce an AITC solution. The treated solution was also used for the root-lesion nematode toxicology study. The flower extract was found to contain sinigrin at a concentration of 66.33µg/ml by the step gradient method previously described. The extract pH was measured as 5.67. The *Brassica* extract was

centrifuged at 3000 rpm for 25 minutes (Sorvall, GLC-1 centrifuge) to remove suspended solids. A stock solution of approximately 1267.6μg/ml myrosinase in pH=6.38 phosphate buffer solution was freshly prepared. *Brassica juncea* extract (40ml) and 10ml of myrosinase stock solution were mixed in a 50ml volumetric flask. The resulting solution was shaken for five minutes and then filtered through a 1.0 micron Acrodisc CR syringe filter (Gelman Sciences). After myrosinase treatment, it was determined that 101% of the sinigrin was converted to AITC (procedure previously described). The resulting solution was then used for the nematode toxicity study.

RESULTS AND DISCUSSION

1. Extraction of sinigrin from *Brassica juncea*

Diffusion extraction involves the solvent extraction of low molecular weight compounds from whole plant tissues. Diffusion extraction has been used for the extraction of glucosinolates from whole *Brassica sp.* seeds[80,85]. In the present investigation, diffusion extraction was used for the extraction of sinigrin from *Brassica juncea* tissues (leaves, flowers and stems). To determine the optimal time necessary to extract sinigrin from *Brassica juncea* (cv. Cutlass) leaves an exhaustive extraction method was used. Exhaustive extraction involves repeatedly extracting a single plant tissue sample. The exhaustive extraction method used for this study involved adding freshly cut *Brassica juncea* leaves to boiling phosphate buffer or methanol/water solutions. A tissue to solvent ratio of 0.03g/ml was used. Aliquotes of the boiling mixture were withdrawn at regular times and analyzed for the sinigrin concentration (see Experimental).

The goal of the diffusion extraction method was to reduce the amount of sinigrin decomposed by endogenous myrosinase. Sinigrin and myrosinase are segregated within *Brassica sp.* tissues[65,71]. Bruising or grinding of fresh *Brassica sp.* tissue can cause significant losses of sinigrin[198]. Myrosinase may be inactivated by temperatures above 70°C[22]. Immersion of rapeseed in boiling water for 1.5 to 3.0 minutes is sufficient to inactivate the endogenous myrosinase[80,84]. Therefore to minimize sinigrin decomposition by myrosinase, whole leaves were carefully cut near the stalk with sharp scissors and immediately plunged into the boiling extraction solvent.

1) Aqueous buffer solution

An exhaustive diffusion extraction of fresh *Brassica juncea* (cv. Cutlass) leaves was performed with a phosphate buffer solution (0.7mM, pH=6.38). A plot of the extracted sinigrin concentration

versus time is shown in **Figure 8**. It should be noted that the concentration data was corrected for by the amount of sample removed from the boiling mixture. Sinigrin was quickly extracted from the whole leaves (**Figure 8**). Within five minutes the equivalent of 1730μg/g (fresh leaf weight) was extracted. Maximal sinigrin extraction occurred during 15 to 35 minutes. The mean and standard deviation values are expressed on the plot (**Figure 8**, 2200±20μg/g). From the results of this study, it can be recommended that the optimal extraction time of sinigrin from *Brassica juncea* (cv. Cutlass) leaves by aqueous phosphate buffer (0.7mM, pH=6.38)is 25 minutes.

After 35 minutes the sinigrin concentration in the extraction mixture was found to steadily decline, presumably because of thermal degradation. The concentration of sinigrin declined linearly during 20 to 60 minutes as follows (**Figure 8**): sinigrin concentration = [-3.5μg/g min.][time(min.)] + [2305μg/g]. This equation implied that the decomposition rate of sinigrin in boiling aqueous phosphate buffer solution was 0.15%/minute.

2) Methanol/water (70:30 v/v)

A similar optimal time of extraction study was performed with methanol/water (70:30 v/v) as the extraction solvent. The initial rate of sinigrin extraction was very fast. The optimal extraction time was determined to be 25 minutes (**Figure 9**). Mean and standard deviation of the maximum sinigrin concentration was 2490±20μg/g (over the range of 20 to 40 minutes). The plant material was selected on the same day and from the same series of plants as the previous aqueous phosphate buffer extraction study (see Experimental). The concentration of sinigrin declined linearly during 30 to 90 minutes as follows (**Figure 9**): sinigrin concentration = [-0.3μg/g min.][time(min.)] + [2488.9μg/g]. This equation implied that the sinigrin decomposition rate in boiling methanol/water (70:30 v/v) solution was 0.01%/minute.

Figure 8: Extraction of *Brassica juncea* (cv. Cutlass) leaves with boiling aqueous phosphate buffer solution

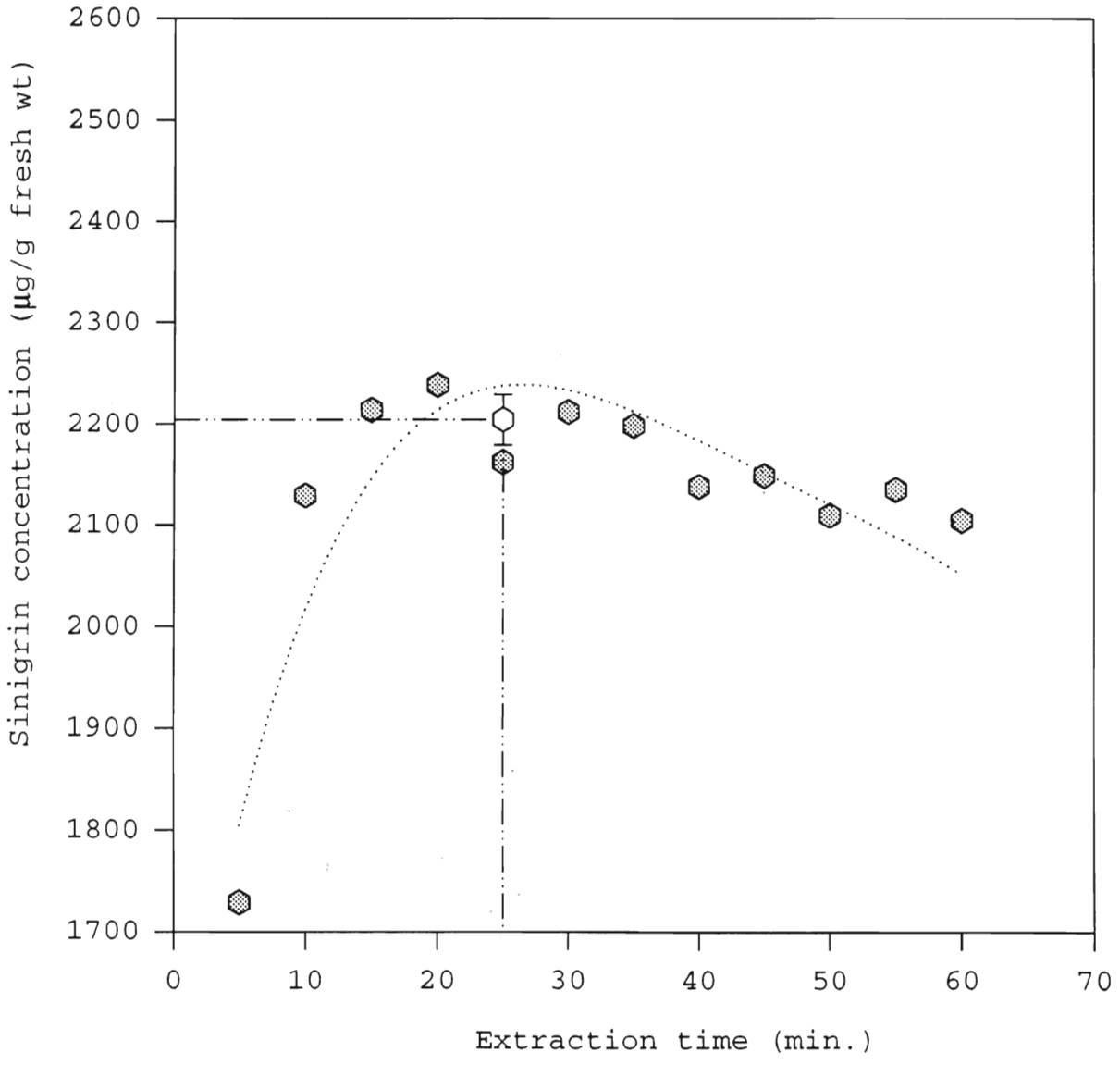
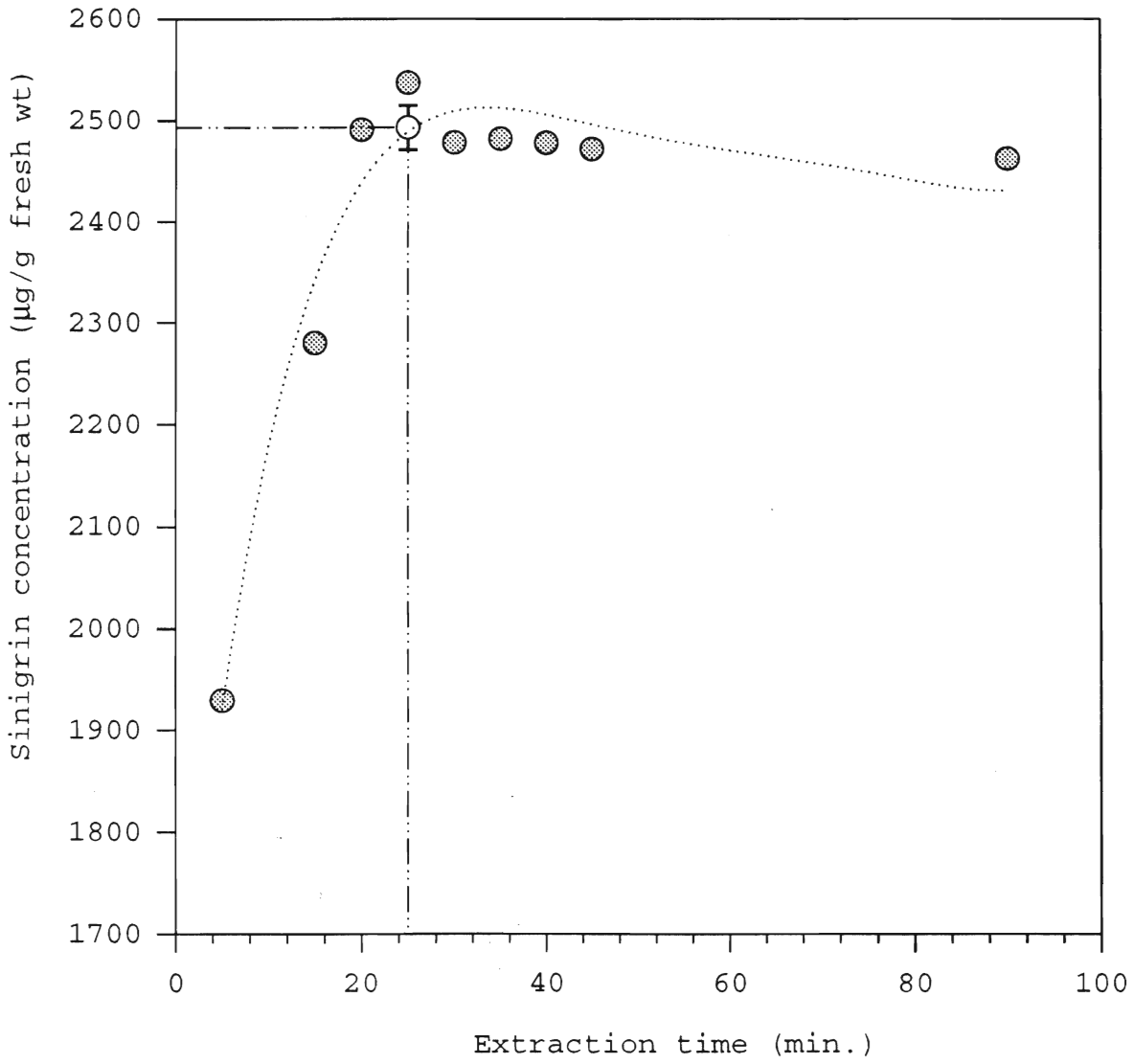


Figure 9: Extraction of *Brassica juncea* (cv. Cutlass) leaves with boiling methanol/water (70:30 v/v) solution



The total amount of sinigrin extracted by the aqueous methanol solvent was 13% greater than the amount extracted by the phosphate buffer solution. This difference can be justified based on the plant material selected (see Experimental). The plants were heat and draught stressed because of growth in a small plant growth chamber (see Experimental). *Brassica sp.* plants may produce a waxy layer on the surface of their leaves when under heat or draught stress[199]. This waxy layer is probably better penetrated by methanol/water (70:30 v/v) solution than the aqueous phosphate buffer solution.

2. Stability of sinigrin in boiling buffer solution

The goal of this study was to determine the stability of sinigrin under the actual conditions which were to be used for sample extraction. Temperatures greater than 110°C can cause decomposition of glucosinolates[22]. Therefore, it can be expected that thermal degradation of sinigrin may occur during diffusion extraction from whole *Brassica juncea* leaves.

The decomposition of sinigrin and other glucosinolates has been studied in aqueous solution[39,43,200,201]. Thermal decomposition of sinigrin in aqueous solution produces mainly allyl-nitrile and sulfate ion[39]. Glucosinolate degradation leads to a release of sulfate ion that causes a decrease in the pH of the solution with time. In 0.1N NaOH sinigrin decomposes ($t_{1/2}$= 18 minutes at 25°C) into thioglucose and vinylglycine ($HO_2C(NH_2)CHCH=CH_2$)[200]. Glucosinolate stability is greatest at pH=7[43,201]. Therefore, extraction solvents should be buffered at approximately pH=7 to minimize sinigrin degradation.

Gronowitz et al. studied the thermal degradation of short alkenyl-glucosinolates in various aqueous solvents[201]. They found a degradation equivalent to 0.48%/minute after 30 minutes of boiling in distilled water[201]. The glucosinolates studied were particularly unstable in borate buffer solution ($Na_2B_4O_7$/NaOH, pH=8)[201]. Almost complete decomposition was observed within 30

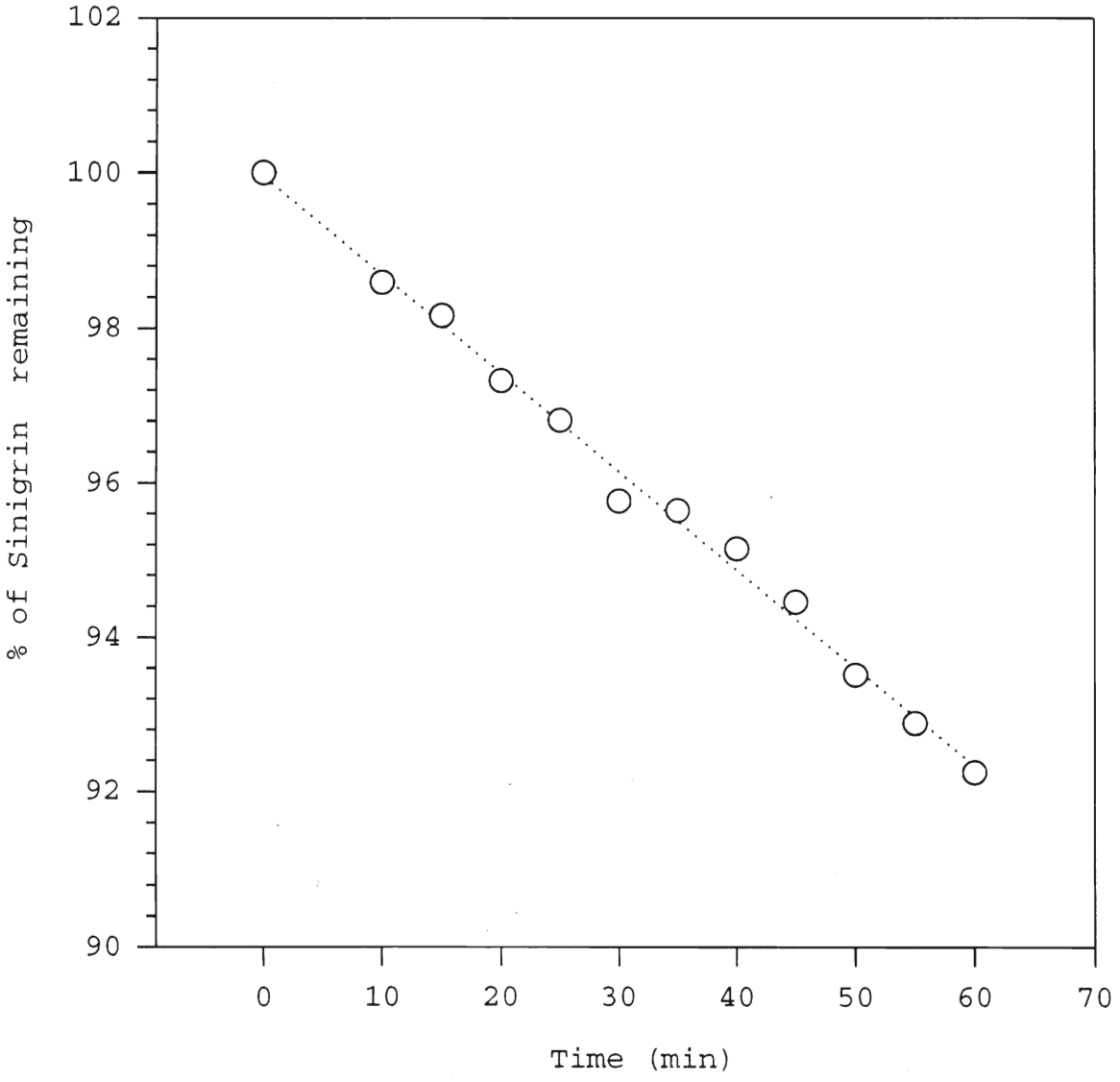
minutes at 100°C in the borate buffer solution whereas 58% was still present when the phosphate buffer at the same pH was used[201]. Therefore, the composition of the aqueous solution can be expected to have a great influence upon glucosinolate stability.

The stability of sinigrin in boiling phosphate buffer solution (0.7mM, pH=6.76) was investigated for this report (**Figure 10**). Percentage decomposition of the sinigrin was measured at regular times. The concentration of sinigrin decreased linearly throughout the study as follows (**Figure 10**): % sinigrin remaining = [-0.13%/min.][time(min.)] + [99.95%]. Therefore, based upon the equation above the loss of sinigrin was approximately 0.13%/minute.

The low rate of sinigrin degradation calculated in this study compares favorably with the results of Maheshwari[40], MacLeod[41] and McLeod and Rossitier[39]. Maheshwari found no decomposition of sinigrin after 30 minutes of heating in pH=5.9 phosphate buffer solution at 100°C. Maheshwari quantified the loss of sinigrin based upon the decrease in UV absorption at 227nm. The stability study method used for this thesis can be considered more accurate than the method of Maheshwari because sinigrin was determined by RP-HPLC-UV (see Experimental). McLeod and Rossitier found that heating a distilled water solution of sinigrin for 30 minutes at 100°C resulted in the equivalent of 0.07% degradation/minute[39]. Sinigrin and its decomposition products were separately quantified[39]. The remaining sinigrin was determined by GC-MS[39].

Our study has suggested that the loss of sinigrin by thermal degradation was approximately 0.13%/minute. Sinigrin can be expected to be stable within the optimal extraction time of 25 minutes using a pH=6.38 phosphate buffer solution. The rate of thermal degradation of sinigrin in boiling phosphate buffer solution (0.13%/minute) was very similar to that observed during the diffusion extraction of a *Brassica juncea* leaf sample by boiling phosphate buffer solution (0.15%/minute). After a 25 minute diffusion extraction of a *Brassica juncea* leaf sample it can be expected that 96.3% of the sinigrin that was originally present

Figure 10: Stability of Sinigrin in boiling aqueous phosphate buffer solution



would remain.

Although it has not been explicitly studied in the literature, the stability of sinigrin in water at room temperature has been estimated to be high[33]. During my research, the decomposition of standard aqueous sinigrin solutions was negligible. Within seven months a peak area deviation of 2% was noted, which was within the experimental error of the method used (RP-HPLC-UV, phosphate buffer mobile phase). The sinigrin solutions were used at room temperature and stored at 1°C at the end of each day.

3. Sinigrin determination by RP-HPLC-UV

During this investigation, various methods for the determination of sinigrin were developed. Below are presented analytical methods that rely upon RP-HPLC with UV absorption detection for the determination of sinigrin.

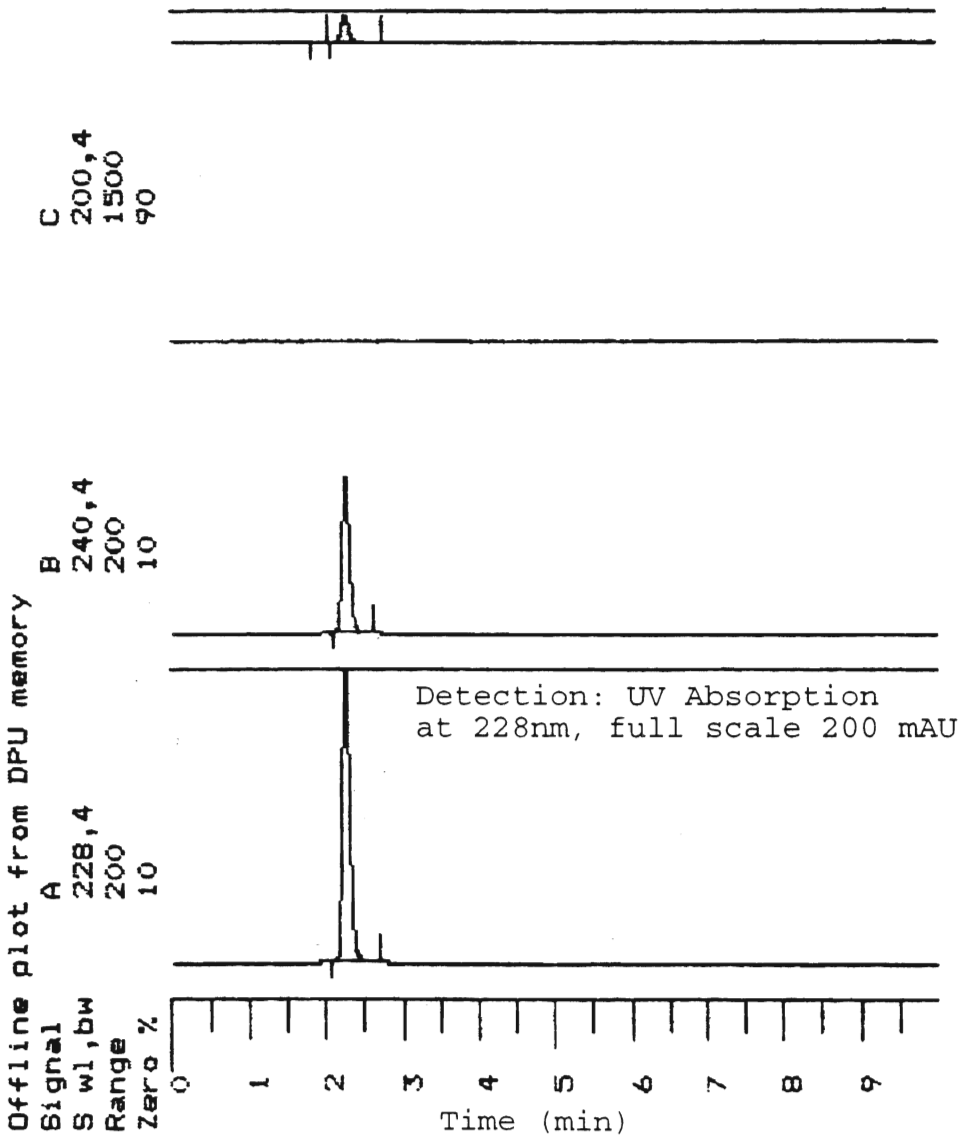
1) Phosphate buffer mobile phase

A fast and simple method for the determination of sinigrin in aqueous solution was required for the above stability study. Sinigrin was determined by a RP-HPLC-UV method using an isocratic phosphate buffer mobile phase. **Figure 11** illustrates a chromatogram resulting from the direct injection of a sinigrin standard (52µg/ml). The method used the same buffer solution as the sinigrin stability study (0.7mM, pH=6.76).

The sinigrin retention time was 2.26 minutes and the system peak occurred at 1.96 minutes (**Figure 11**). System peak assignment was based upon the retention time of CTAB. The sinigrin peak width was 0.10 minutes at half height. Total analysis time was under three minutes.

Figure 11: Determination of sinigrin by RP-HPLC-UV using an isocratic phosphate buffer mobile phase

(for further details see Experimental, Results and Discussion)



The number of effective theoretical plates (N) was determined by the following formula[202]:

$N = -8 \ln(1/2)(t_R'/W_h)^2$
where, t_R' is the adjusted retention time $t_R' = t_R - t_0$
t_R is the retention time of the compound
t_0 is the retention time of the system peak
W_h is the width of the peak at half the height

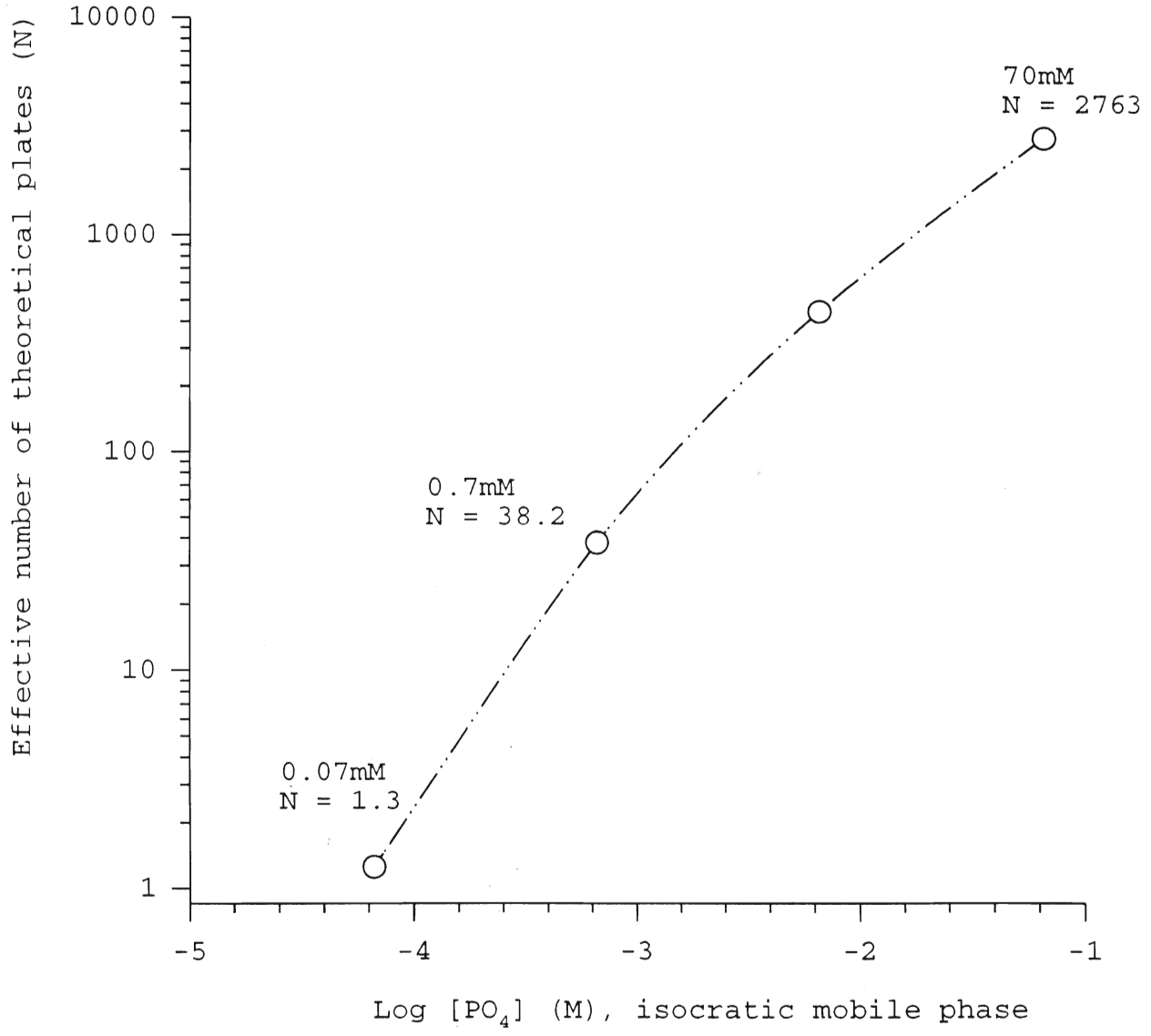
Using the above equation, the effective number of theoretical plates for the separation (**Figure 11**) was 38.2. This method should only be used for the determination of sinigrin in pure samples because of the low theoretical plate number.

The analytical procedure developed for this study was similar to that used by Bjorkqvist and Hase[113]. The concentration of phosphate buffer salt employed was approximately 150 times lower than that recommended by Bjorkqvist and Hase[113]. The low concentration of phosphate buffer was sufficient to give a separation of sinigrin from the system peak (**Figure 11**). Purity of the sinigrin peak was monitored by comparison of the UV spectra acquired on the apex and base with a standard spectrum (**Appendix 8**).

Bjorkqvist and Hase recommend that a mobile phase buffer salt concentration of 0.1M should be used for the analysis of glucosinolate mixtures by RP-HPLC. For this work, a buffer salt concentration of 0.7mM was used to allow for rapid analysis. Based upon the expected sample purity and composition, use of a low buffer salt concentration was acceptable. One consequence associated with the use of a high buffer salt concentration in the mobile phase is short pump seal life[203]. Buffer solution concentrations more than 0.1M are particularly trouble prone[203].

For this thesis, the effect of phosphate buffer salt concentration (isocratic mobile phase) upon the retention of sinigrin was studied. **Figure 12** displays the effective number of

Figure 12: Effective number of theoretical plates (N) for sinigrin versus the concentration of phosphate buffer salt in the isocratic mobile phase



theoretical plates as a function of the concentration of phosphate salts in the isocratic mobile phase. Notice that the number of theoretical plates increased with the concentration of phosphate buffer salts in the mobile phase.

The pH of the mobile phase was maintained at approximately seven. The effect of mobile phase pH upon the retention time of sinigrin was not studied because sinigrin is a strong acid[43]. Sinigrin retention time was not expected to be altered by mobile phase within the pH range commonly used with C_{18} chemically bonded silica stationary phases (pH=2.0 to 7.5)[204,205].

2) Acetate buffer mobile phase

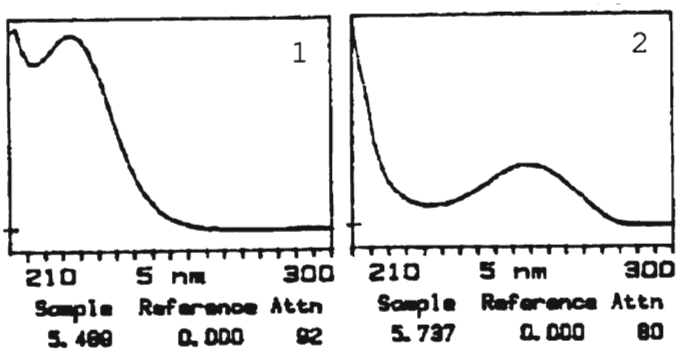
A RP-HPLC-UV method was developed for the determination of sinigrin in aqueous *Brassica juncea* extracts. The method employed an isocratic 0.1M ammonium acetate mobile phase (**Figure 13**) similar to previously published methods[113,114]. Sinigrin retention time (**Figure 13, peak #1**) was 5.49 minutes. The UV absorption spectrum of sinigrin (**Figure 13, spectrum #1**) was identical with the spectrum of pure sinigrin (**Appendix 8**). Ammonium acetate mobile phase offers the advantage of ease of preparation. The mobile phase is prepared by simply dissolving the ammonium acetate in water and then vacuum degassing/filtering.

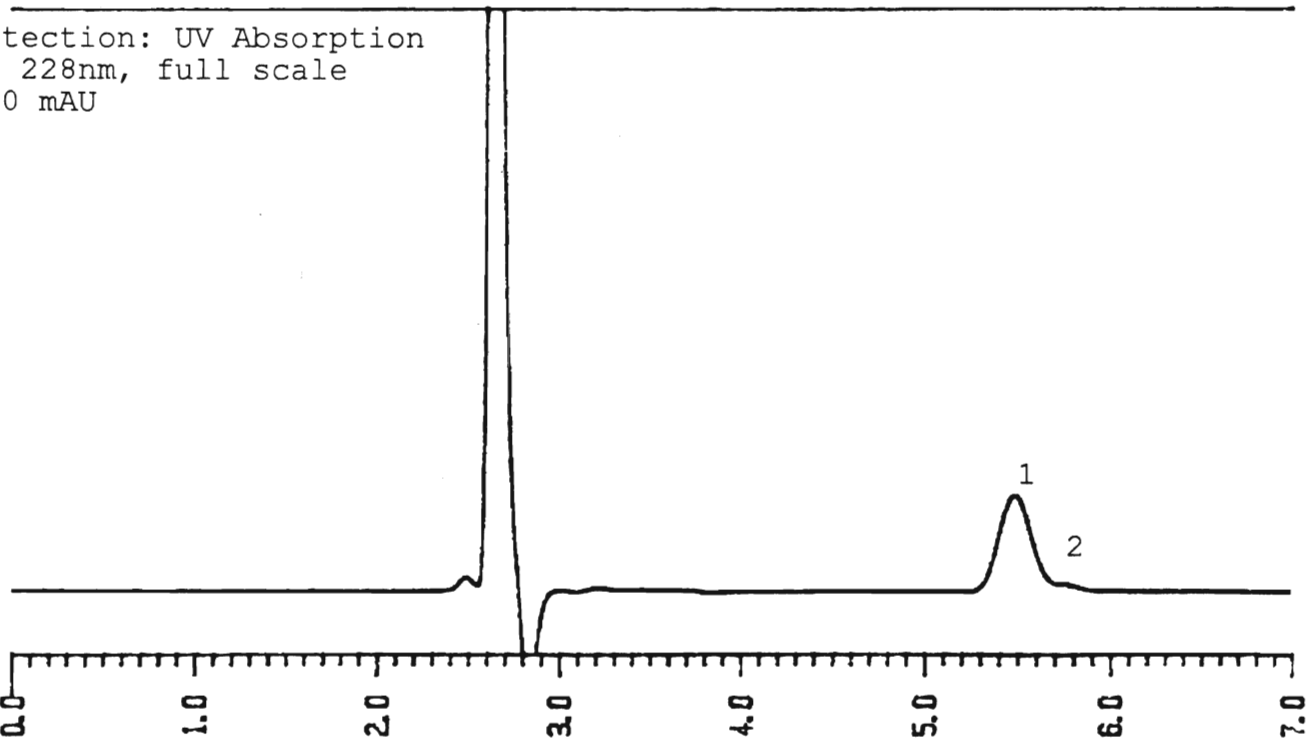
A *Brassica juncea* (cv. Cutlass) leaf extract harvested at the third week of growth was found to contain the greatest concentration of impurities (**Figure 13, peak #2**). The impurity (**Figure 13, peak #2**) was eluted on the far tail end of the sinigrin peak. Notice that the acquired UV spectrum of the impurity (**Figure 13, spectrum #2**) had a local minimum of absorbance near the absorption maximum of sinigrin (**Figure 13, spectrum #1**). The interference of **peak #2** (**Figure 13**) with sinigrin determination can be expected to be quite minimal.

Figure 13: Chromatogram of an aqueous phosphate buffer *Brassica juncea* (cv. Cutlass) leaf extract using an ammonium acetate mobile phase

(for further details see Experimental, Results and Discussion)

UV Spectra:





3) Ion-pair mobile phase

Shown in **Figure 14** is a chromatogram of the separation of a *Brassica juncea* (cv. Cutlass) leaf extract [methanol/water (70:30 v/v)]. A CTAB containing isocratic mobile phase was used for the separation, 22% acetonitrile/63% 0.1M ammonium acetate/15% 1mM CTAB. The developed method employed a mobile phase flow of 700µl/minute. The method used a very low concentration of CTAB in the mobile phase (150µM). The concentration of CTAB was much lower than that used by other researchers[74,92,93,112]. Generally, ion-pair separations use mobile phase CTAB concentrations between 25mM[206] and 5mM[207]. For this investigation, a low concentration of CTAB in the mobile phase was used to reduce the adverse effect of the ion-pair reagent on the analytical column.

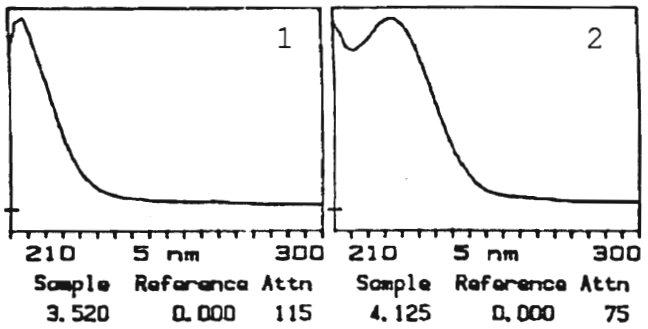
Knox and Hartwick showed that alkyl-sulfonate ion-pair reagents irreversibly bind and decompose reversed-phase HPLC stationary phases (C_{18} chemically bonded silica)[208]. They found that the desorption rate (from C_{18} chemically bonded silica) of alkyl-sulfonate ion-pair reagents (C_8-C_{12}) decreases as the alkyl chain length increases[208]. An analytical column is often irreversibly altered if a strongly adsorbed ion-pairing reagent is used. For example, they found that they were unable to wash sodium dodecyl-sulfate (SDS, $CH_3(CH_2)_{11}OSO_3Na$) from an analytical column even with 100% methanol or isopropanol[208]. Even small amounts of SDS can deteriorate the performance of reversed phase columns leading to lower resolution and altered retention times[209]. Tetra-alkyl-ammonium ion-pair reagents have also been reported to degrade silica-based stationary phases[111].

During this investigation, the deterioration of a C_{18} (chemically bonded silica) reversed-phase column was observed after repeated use of an alkyl-ammonium halide (tetra-heptyl-ammonium bromide, 5mM) containing mobile phase. Deterioration of the stationary phase was observed after approximately three months of use. A waxy solid was collected during the dichloromethane washing stage while attempting the regeneration[210] of the poorly performing

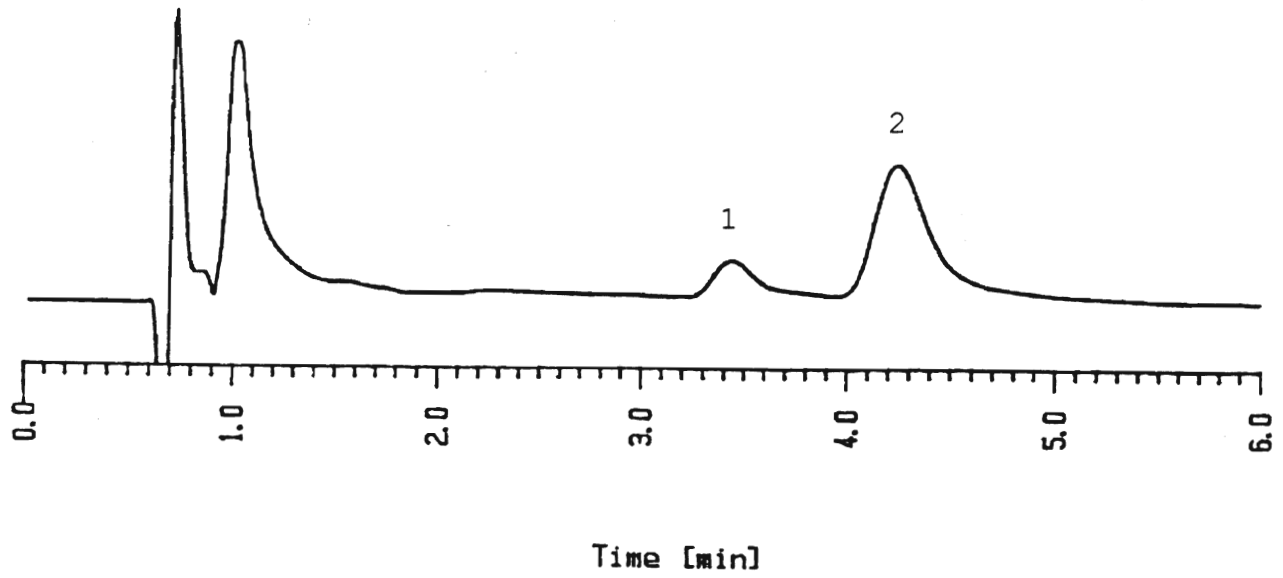
Figure 14: Chromatogram of a methanol/water (70:30 v/v) *Brassica juncea* (cv. Cutlass) leaf extract using an ion-pair mobile phase

(for further details see Experimental, Results and Discussion)

UV Spectra:



Detection: UV Absorption
at 228nm, full scale
500 mAU



analytical column. The waxy solid was identified by electron impact mass spectrometry (EI-MS) as a complex organo-silicone containing mixture.

A problem associated with the use of a low concentration of CTAB in the mobile phase was the dependence of the sinigrin retention time upon the sample composition. The effect of sample composition upon sinigrin retention time was quite noticeable. After repeated injections of a *Brassica juncea* extract prepared in 70% methanol the sinigrin retention time was approximately 4.13 minutes. Methanol in the sample causes desorption of the ion-pair reagent from the stationary phase[207]. Injection of sinigrin prepared in 100% water caused progressively longer retention time with each new injection. For the current work, samples prepared in 100% water caused a gradual increase in sinigrin retention time, because of the accumulation of CTAB (from the mobile phase) on the stationary phase.

Another problem associated with CTAB was the tendency for its aqueous solutions to form persistent foam and bubbles. Therefore, mobile phases containing CTAB could not be vacuum degassed and filtered. Also, long equilibration times are required when ion-pair reagent mobile phases are used (often hours)[207]. For this thesis, the long equilibration time made method development a very time-consuming process.

A favorable aspect of the CTAB separation method was that 70% methanol *Brassica juncea* extracts were analyzed directly without clean up (other than syringe tip filtration). The UV absorption spectra in **Figure 14** show spectra acquired at the apex of each peak. These UV absorption spectra show that the sinigrin peak (**Figure 14, peak #2**) is baseline separated from impurities. **Spectrum #2** (**Figure 14**) is identical with the UV absorption spectrum of pure sinigrin (**Appendix 8**).

4) Step elution method

An interesting separation method was developed. The major discovery was that an analytical column needs only to be preconditioned with phosphate buffer solution to separate sinigrin from CTAB (the unretained compound). After preconditioning, the mobile phase was switched to 100% water at the time of injection for subsequent elution of sinigrin.

Figure 15 illustrates a chromatogram of a *Brassica juncea* (cv. Cutlass) leaf extract (harvested at week three, cleaned up by ion-pair SPE) separated by a phosphate buffer solution/water step gradient system. The method involved a five minute conditioning of a 25cm C_{18} analytical column (and 2cm C_{18} precolumn) with 5mM phosphate buffer solution. At the time of sample injection the mobile phase was switched to 100% water. Sinigrin was eluted at 2.81 minutes with a peak width (half height) of 0.035 minutes (**Figure 15, peak #1**). The effective number of theoretical plates was 2237.1. The retention time for the unretained component (CTAB) was 2.12 minutes which, was determined by a separate injection of CTAB.

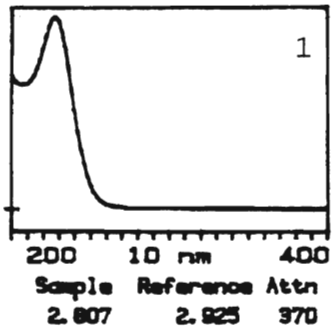
The effective number of theoretical plates for sinigrin versus the concentration of phosphate salt in the conditioning mobile phase was studied and plotted in **Figure 16**. It should be noted that the number of theoretical plates increased rapidly with higher concentrations of phosphate salt in the conditioning mobile phase. This method worked well at low phosphate salt concentrations because the sinigrin peak width was very sharp (**Figure 15**).

For this research, a phosphate salt concentration of 0.05M was used for subsequent investigations. A very desirable aspect of this method was that the phosphate salts are washed from the system during each analysis cycle. Thus, the potential for the clogging of the HPLC capillaries was greatly reduced. Also, column life can be extended because phosphate buffer salts, which may precipitate and cause increased back pressure, were eliminated.

Figure 15: Chromatogram of an aqueous phosphate buffer *Brassica juncea* (cv. Cutlass) leaf extract separated by a phosphate buffer step gradient system

(for further details see Experimental, Results and Discussion)

UV Spectrum:



Detection: UV Absorption
at 228nm, full scale
1000 mAU

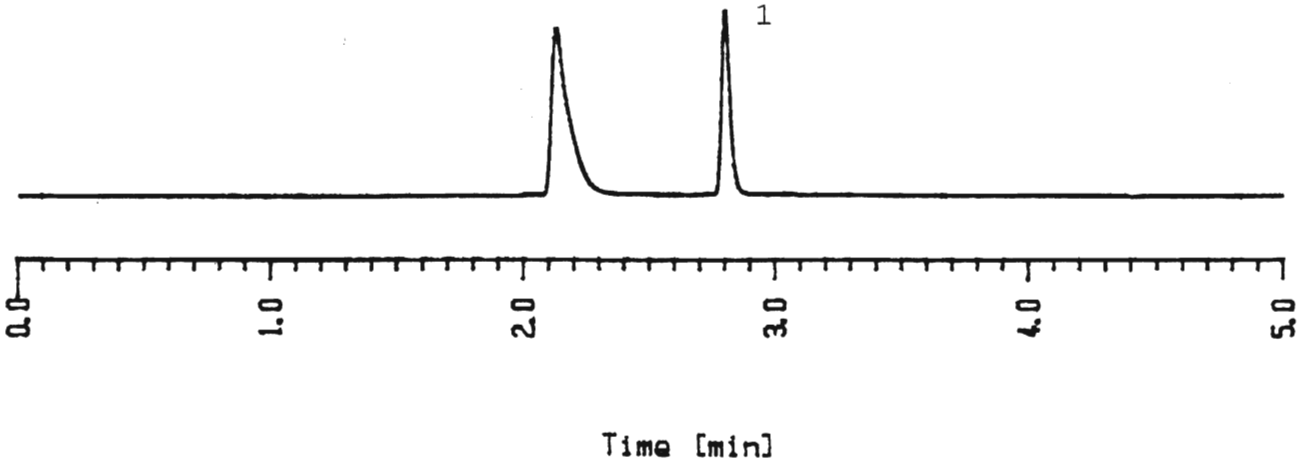
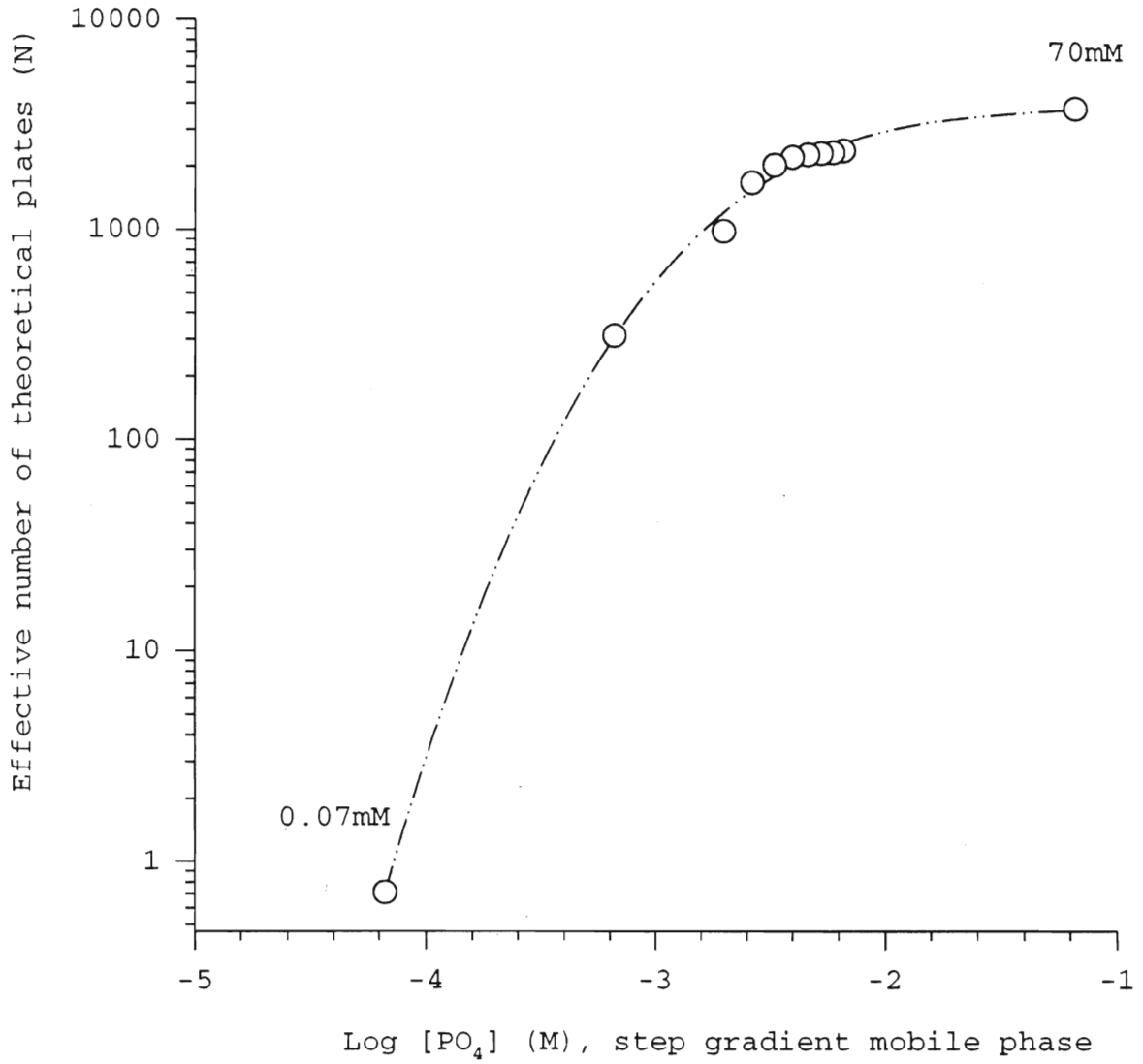


Figure 16: Effective number of theoretical plates for sinigrin versus the concentration of phosphate salt in the conditioning mobile phase



4. Sinigrin and benzyl-glucosinolate determination by HPLC-PB-NCI-MS

The first NCI mass spectra of glucosinolates are illustrated in **Figure 17.** Sinigrin and benzyl-glucosinolate were compared to determine the common glucosinolate fragmentation pathway. The PB interface was used to introduce the sample into the mass spectrometer and methanol was used as the chemical ionization reagent gas (see Experimental). The methanol was ionized by an EI mechanism in the MS source to give methoxide (CH_3O^-).

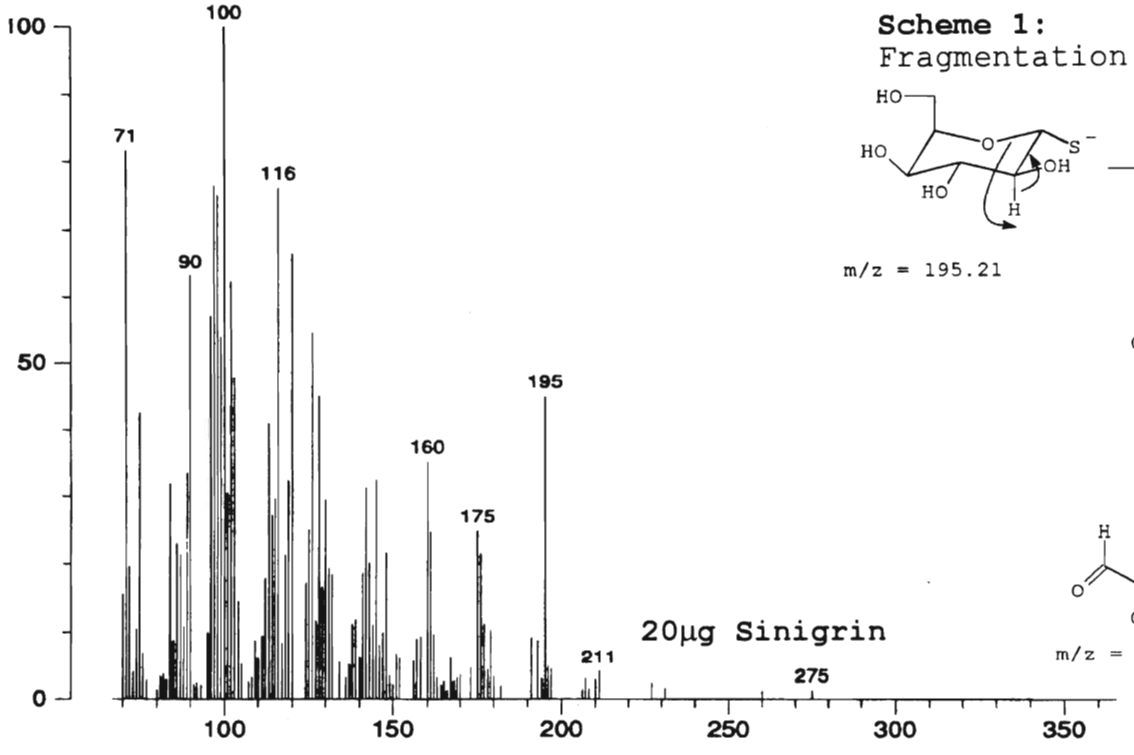
The (CH_3O^-) NCI mechanism involves molecular anion formation by abstraction of a proton with the strong Bronsted base methoxide[211]. The molecular anion $[M-H]^-$ can be expected to be quite stable to fragmentation because most of the energy resides with the formation of the new bond in methanol[211]. Consequently, fragmentation is much more limited in NCI than with PCI or EI mechanisms.

For the current work, it is proposed that the m/z=195 fragment arise from cleavage of the thioglucose moiety from the glucosinolate molecule. A degradation scheme for thioglucose is proposed (**Figure 17, scheme 1**); the thioxy anion form of thioglucose rearranges into the thio-enolate. The thio-enolate anion could then fragment by a McLafferty rearrangement[212] to a fragment at m/z=105. The scheme is similar to the negative ion fragmentation pathway for glucose proposed by Hunt et al[211].

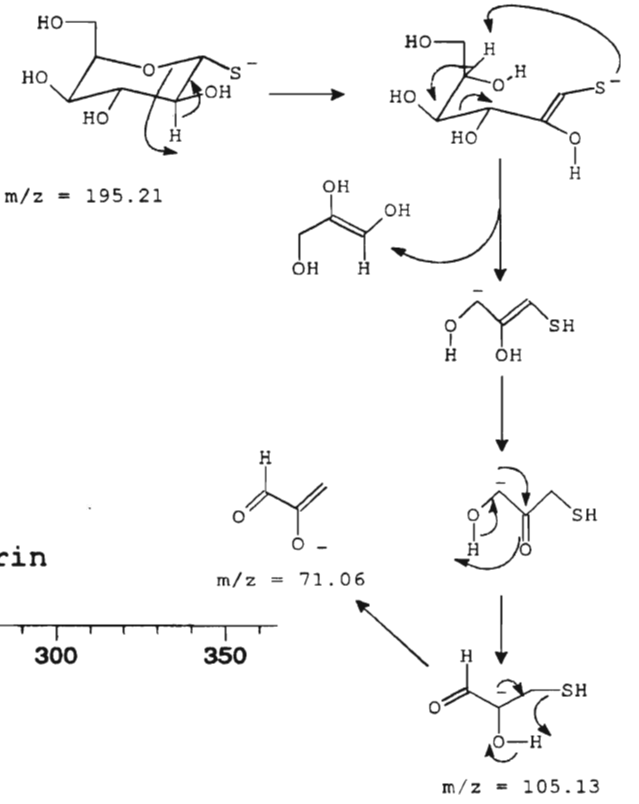
From a series of ketone anions, generated by methoxide as a reagent gas, the primary fragmentation mechanism was McLafferty rearrangement[213]. Migration of a γ-hydrogen to the carbanion (α to the carbonyl group) occurs through a cyclic transition state containing six atoms[213]. Referring to **Figure 17, scheme 1;** the thioglucose derived fragment at m/z=105 underwent loss of hydrogen sulfide (H_2S) to give a species at m/z=71. The m/z=71 fragment is a dominant peak observed in the spectra of both sinigrin and benzyl-glucosinolate.

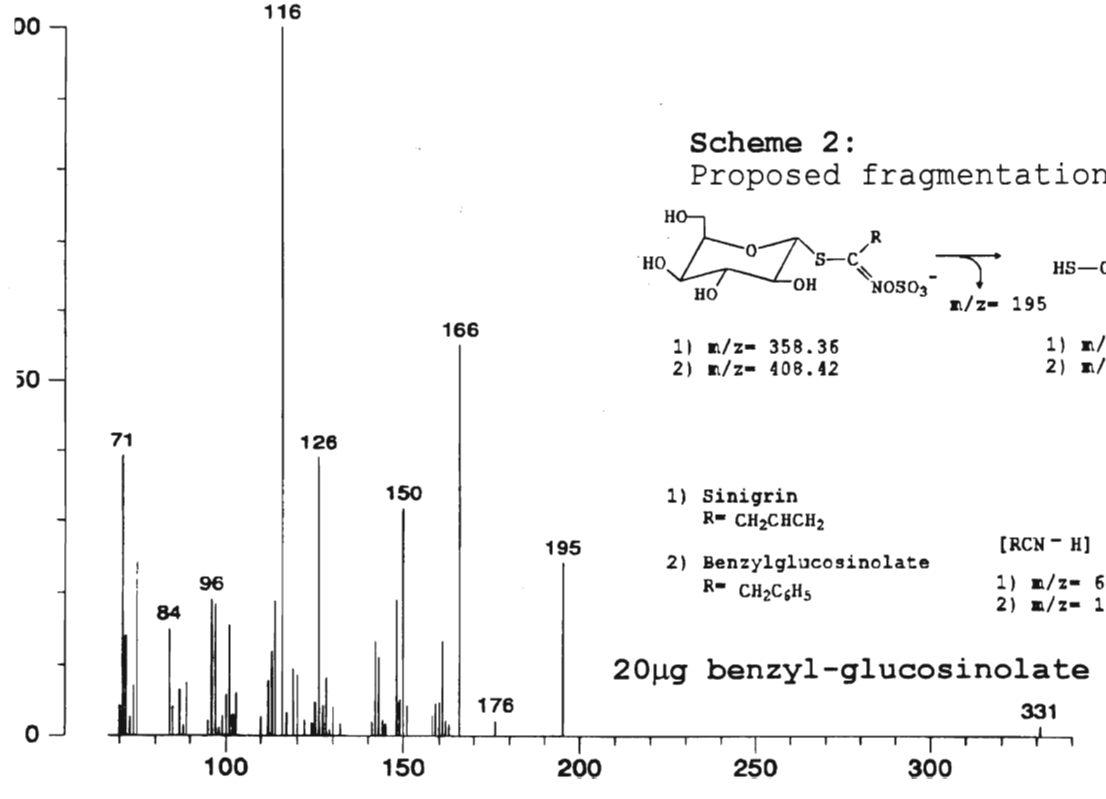
Figure 17: Negative chemical ionization mass spectra of sinigrin and benzyl-glucosinolate

(for further details see Experimental, Results and Discussion)



Scheme 1:
Fragmentation of thioglucose





Scheme 2:
Proposed fragmentation of glucosinolates

A feature of the HPLC-PB-NCI-MS mass spectra of both sinigrin and benzyl-glucosinolate was the abundance of even molecular weight fragments. This suggests by the "nitrogen rule"[214] that these fragments are even electron (EE^-) species containing an odd number of nitrogens. Major low mass fragments were explained in **Figure 17, scheme 2**. The scheme was constructed by expecting the loss of labile sulfur trioxide or sulfur dioxide, oxygen and hydrogen sulfide.

It is well known that artifacts can be present in NCI-MS spectra. Artifacts are produced by radical reactions and adduct ion formation[215]. There is greater tendency for thermally induced reactions than under EI conditions; these reactions include dehydration, labile group losses, and rearrangements[215]. It is expected that NCI spectra and artifact formation are very sensitive to ionization conditions[215]. Experiments should be conducted to optimize the ionization parameters.

The base peak in the benzyl-glucosinolate spectrum was the benzyl-nitrile fragment $[RCN-H]^-$ at m/z=116. Unfortunately, the allyl-nitrile fragment (m/z=66) was not observed in the sinigrin spectrum because experimental parameters were not set to observe the low m/z value. Nethertheless, HPLC-PB-NCI-MS should be useful for glucosinolate determination. Glucosinolate side chain structure could potentially be identified from the observed alkyl-nitrile fragment.

Of the commonly used reagent gases, hydroxide (OH^-) is the strongest gas phase Bronsted base[215]. There may be some utility with trying OH^- as a reagent gas to determine the analyte molecular weight. For example, hydroxide NCI-MS was very effective for $[M-H]^-$ formation from polysorbates (polyoxyethylated fatty acid esters of sorbitans)[216]. Hydroxide can be produced from nitrous oxide (N_2O) and methane in the source region[215].

Chloride (Cl^-) is a reagent gas that may find some utility in

glucosinolate analysis. Chloride is a weak gas phase base[215]. There is a possibility that molecular weight information can be directly found from the use of chloride as a reagent gas. Chloride is known to react with acidic substrates (such as carboxylic acids and polyhydroxyl compounds) by attachment[215]. Limited fragmentation is expected with the attachment reaction[215]. Using chloride as the reagent gas, the NCI-MS spectra of glucose displayed the adduct $[M+Cl]^-$ as the main peak observed[215]. Mixture analysis by HPLC-PB-NCI-MS may be possible if each component gave a single ion characteristic of each respective molecular weight[211].

Based upon the results of this research, NCI-MS is equally sensitive to both sinigrin and benzyl-glucosinolate if the thioglucose fragment (m/z=195) is considered. Separate injections of sinigrin and benzyl-glucosinolate (20μg) gave approximately the same count level for the m/z=195 peak (97223 and 81867 counts, respectively). But, intensity of comparable fragments at m/z=100 (sinigrin) and m/z=150 (benzyl-glucosinolate) are quite different (290972 and 107931 counts respectively). For sinigrin, isobutane was also tried as a reagent gas but no peaks were observed. HPLC-PB-PCI-MS of sinigrin with methanol as a reagent gas was also tried, but the technique was very insensitive (the only ion detected near the noise floor was m/z=80).

There is a possibility that the HPLC-PB-NCI-MS technique could be developed as a fast general technique for the determinations of the total glucosinolate concentration in *Brassica sp.* tissues. Possibly, the total glucosinolate concentration could be determined by monitoring the thioglucose anion (m/z=195). Experiments should be conducted on a series of glucosinolates to see if response at m/z=195 is the same for all glucosinolates.

Potentially, HPLC-PB-NCI-MS could be used for the determination of glucosinolates in crude *Brassica sp.* extracts. Negative mode FAB has been applied to the analysis of a crude *Brassica sp.* seed paste[127]. Individual glucosinolates were identified by the molecular anions[127]. HPLC-PB-NCI-MS could also be

used in a similar manner except individual glucosinolate concentrations may be determined by monitoring the $[RCN-H]^-$ fragments. Alternatively, the total glucosinolate concentration could be determined by monitoring the thioglucose anion (m/z=195). Experiments should be conducted to determine if interfering levels of endogenous thioglucose are present in *Brassica sp.* tissues. Also, it would be important to determine if direct sample introduction has a severe fouling effect in the source region of the mass spectrometer.

The HPLC-PB-NCI-MS apparatus was easy to setup. Generally, the solvent vacuum skimming was allowed to stabilize for approximately one hour before the start of experiments. The technique was fast and sample introduction via the PB interface can be repeated approximately every minute.

5. *Brassica juncea* extract cleanup

Reversed-phase chromatography of a directly injected aqueous *Brassica juncea* extract was explored. Preliminary studies with a phosphate buffer mobile phase (0.01M, pH=7) revealed the presence of coextractives in the extract that was eluted at the same retention time as sinigrin. Various compounds have been suggested as possible coextractives: sinapine, lipids, carbohydrates and proteins[75]. An ion-pair SPE sample clean up method was developed to separate sinigrin from the coextractives and to protect the analytical column.

Ion-pair SPE is a sample clean up technique used for aromatic sulfonic acid[217], pesticide[218] and glucosinolate[78] determinations. Betz and Fox developed an ion-pair SPE clean up method using a C_{18} chemically bonded silica stationary phase and TBAS as the ion-pair reagent[78]. The *Brassica sp.* extract (5ml) was loaded onto the SPE cartridge. Then, the SPE cartridge was washed with 1ml of water[78]. Glucosinolate elution was accomplished by washing the SPE cartridge with 1ml of methanol/water solution (55:45 v/v)[78]. The ion-pair SPE sample clean up method presented in this report (see Experimental,

Figure 7) was developed independently of the recent published report by Betz and Fox[78].

The cleanup method developed for this thesis differs from the method of Betz and Fox[78] in several respects. First, Betz and Fox employed a rinsing step of 1ml water[78]. In the present work a rinsing step of 9ml acetonitrile/1mM CTAB (15:85 v/v) was used. Sinigrin and benzyl-glucosinolate were retained on the solid sorbent under these rinsing conditions (**Table 2A**). The method also differs from that of Betz and Fox in that glucosinolate elution was accomplished by 5ml acetonitrile/water (50:50 v/v) instead of 1ml of methanol/water (55:45 v/v)[78].

Sinigrin breakthrough during sample loading was not observed with the current method (**Table 2A**). To prevent analyte breakthrough CTAB was added to the sample (final concentration was 1mM). Betz and Fox did not add the ion-pair reagent to the *Brassica sp.* extract[78]. It should be noted that the major proportion of coextractives was eliminated during the sample loading step (80.5% of the total coextractive amount, **Table 2B**). The remainder of the coextractives was eliminated by the sample rinsing step before the elution of the analyte (**Table 2B**). Acetonitrile concentration in the analyte elution step was limited to minimize baseline disturbance during HPLC analysis of the cleaned up sample. In contrast, Betz and Fox found that high organic solvent concentrations in the analyte elution step increased glucosinolate recovery[78]. Unfortunately, they also found the interference from coextractives was also increased[78].

The developed cleanup proved to be efficient for the determination of sinigrin without any interference from the coextractive impurities. This judgement was based upon comparison of the acquired UV absorption spectrum with pure sinigrin (**Appendix 8**). The UV absorption spectrum of the coextractive was substantially different from that of sinigrin. With the present method essentially quantitative recoveries of sinigrin and benzyl-glucosinolate were accomplished (92.6% and 87.4% respectively,

Table 2: Percentage recovery of Sinigrin in the fractions from ion-pair SPE clean up

A: Sinigrin and benzyl-glucosinolate standards prepared in aqueous 1mM CTAB

	SPE Fraction	**% Glucosinolate recovery**
1	**Wash**: 2x6ml Methanol 2x6ml 1mM CTAB	NA
2	**Sample loading**: 20ml standard prepared in aqueous 1mM CTAB	---
3	**Rinsing**: 9ml ACN/1mM CTAB (15:85 v/v)	---
4	**Analyte elution**: 5ml ACN/water (50:50 v/v)	92.6% (sinigrin) 87.4% (benzyl-glucosinolate)
5	**Analyte elution**: 5ml ACN/water (50:50 v/v)	---

B: Aqueous *Brassica juncea* extract prepared in 1mM CTAB

	SPE Fraction	**% of total coextractives★ eluted**
1	**Wash:** 2x6ml Methanol 2x6ml 1mM CTAB	NA
2	**Sample loading:** 20ml aqueous *Brassica juncea* extract prepared in 1mM CTAB	80.5%
3	**Rinsing:** 9ml ACN/1mM CTAB (15:85 v/v)	19.5%
4	**Analyte elution:** 5ml ACN/water (50:50 v/v)	--- (sinigrin eluted)
5	**Analyte elution:** 5ml ACN/water (50:50 v/v)	---

NA not applicable
--- peaks not detected
★ eluted at the retention time of sinigrin

Table 2A). A second repeated eluent from the SPE cartridge did not contain any sinigrin (**Table 2A**). The current method is substantially better than that presented by Betz and Fox in which the average glucosinolate recovery was 85%[78].

The ion-pair method presented in this report resulted in the complete separation of sinigrin from coextractive impurities in high yield. The method is unique because the sample rinsing solvent contains acetonitrile and CTAB. Glucosinolates are retained by the presence of the ion-pair reagent. The high concentration of acetonitrile in the rinsing solvent allows coextractive impurities to be removed. Potentially, the developed method can be extended to the clean up of *Brassica sp.* extracts containing other glucosinolates.

6. Determination of sinigrin in *Brassica juncea*

1) Plant tissue sampling

When selecting plant tissues, it is important to take a representative sample of the crop. During this investigation, leaves were taken from the same position on each plant. Insect damaged, diseased and mechanically injured leaves were not sampled. Also, only plants at the same maturity stage were sampled. For elemental analysis leaves from 10 to 100 plants are needed to give a representative sample[219]. For this report a series of four replicates with leaves from approximately five plants were taken for each sampling. The stability of *Brassica sp.* tissues and extracts under various conditions are not clear[198]. For example, there are conflicting reports in the literature about the stability of glucosinolates in frozen *Brassica sp.* tissues[78,220]. Therefore, during this investigation tissues and extracts were used immediately without storage.

2) Variation with plant age

The sinigrin concentration in the upper leaves of *Brassica juncea* (cultivars Cutlass and Domo) was determined over the life of the plant (**Figures 18** and **19**). The sinigrin concentration in the cotyledons at germination was 470±90µg/g and 600±100µg/g for Cutlass and Domo respectively (fresh weight basis, **Figure 18** and **19**). The leaf sinigrin concentration for Cutlass declined to a minimum value at the third week after planting (200±100µg/g, **Figure 18**). Similarly, the minimum sinigrin concentration in the Domo cultivar occurred at the fourth week after planting (190±70µg/g, **Figure 19**). The mean sinigrin concentration in the plant tissue was found to increase from the fourth through eighth week (**Figures 18** and **19**). Maximum sinigrin concentration in Cutlass occurred at the seventh week (1300±200µg/g, **Figure 18**). Domo displayed a maximum sinigrin concentration at the eighth week (1100±400µg/g, **Figure 19**). Furthermore, the overall weekly percentage variablity of sinigrin concentration is 30±10% and 40±10% for Cutlass and Domo, respectively (**Figures 18** and **19**).

Few researchers have studied the change in plant tissue sinigrin concentration during the growth of *Brassica juncea* plants[77]. In contrast, many reports have been published describing the change in short chain alkenyl-glucosinolate concentrations during *Brassica napus* growth[221,222,223]. *Brassica juncea* and *Brassica napus* are closely related species with *Brassica campestris* as the common parent[18]. *Brassica napus* tissue is characterized mainly by the short chain alkenyl-glucosinolates 3-butenyl-glucosinolate and sinigrin[18,44,221,223]. *Brassica juncea* leaf tissues are characterized by a single glucosinolate, sinigrin[22,23,42]. Also, 92.9% of the total glucosinolate content of *Brassica juncea* seed is reported to be sinigrin[82]. It is of interest to compare sinigrin concentration changes during the growth of *Brassica juncea* with published accounts of short chain alkenyl-glucosinolate changes during the growth of *Brassica napus*.

Figure 18: Sinigrin concentration in the top three leaves of *Brassica juncea* cv. Cutlass

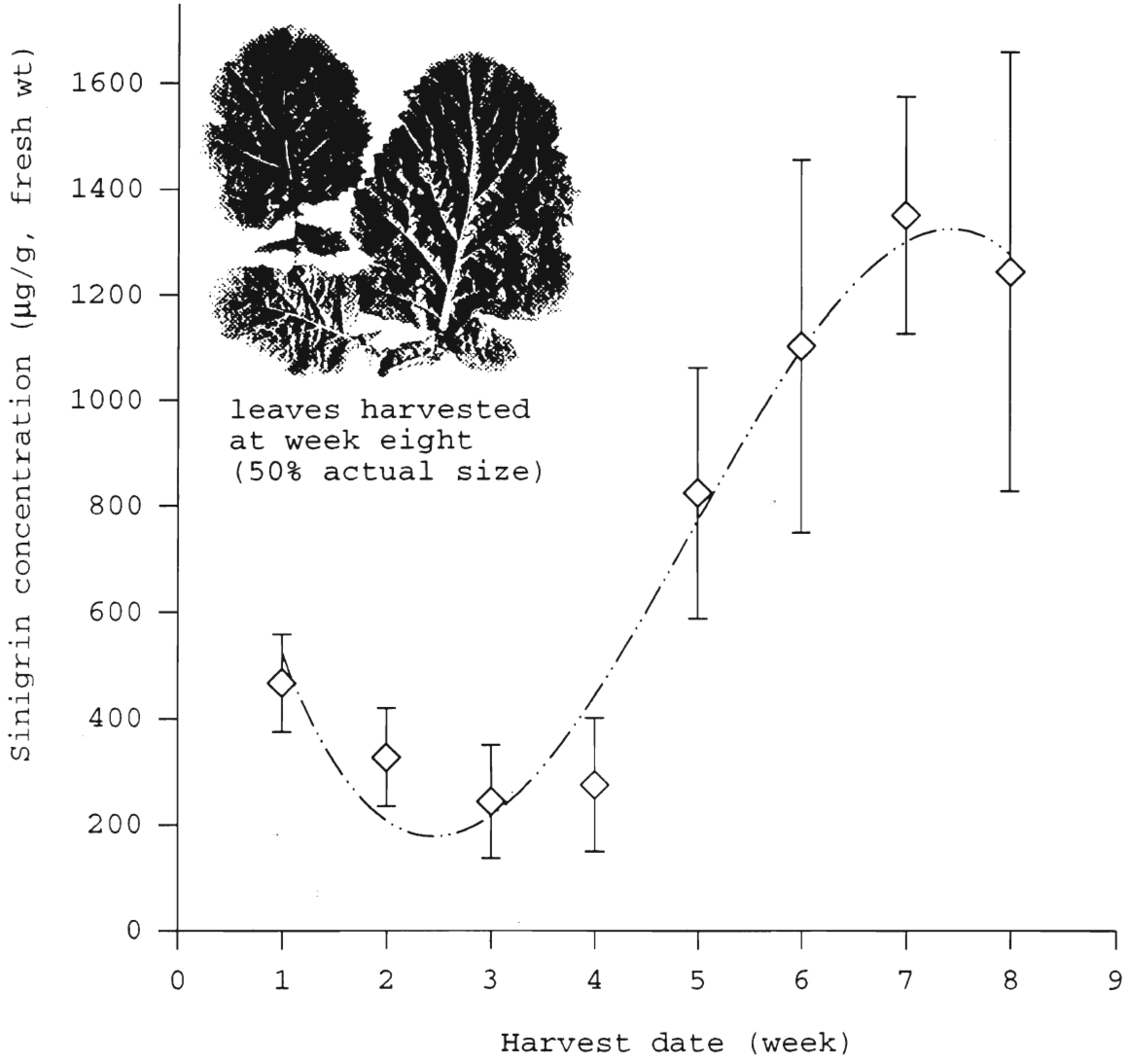
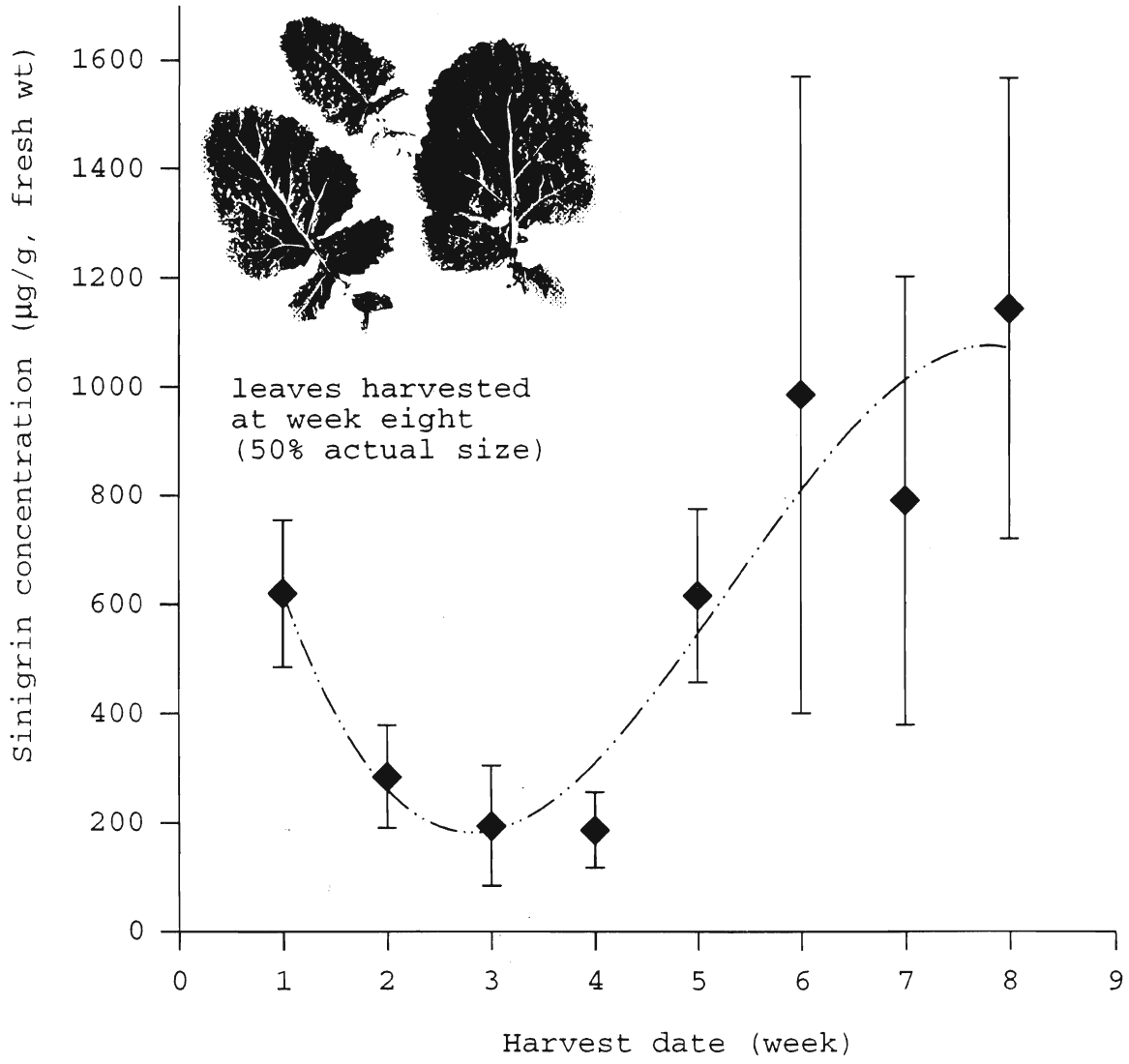


Figure 19: Sinigrin concentration in the top three leaves of *Brassica juncea* cv. Domo



Bodnaryk and Palaniswamy studied the changes of glucosinolate concentration in *Brassica juncea* (cv. Cutlass) cotyledons during plant growth[77]. They found a fivefold decrease in the concentration of sinigrin in cotyledon tissues between the second and fourteenth days after planting[77]. This decrease in the sinigrin level of the cotyledons was proposed to be due to the dilution of sinigrin in the tissue as the plant mass increased with age[77]. The highest sinigrin concentration was observed at germination (approximately two days after seeding); 2150µg/g dry tissue[77].

Bodnaryk and Palaniswamy found that the sinigrin concentration decreased as the sulfate salt concentration in the growth medium was decreased[77]. But, the initial sinigrin concentration in the cotyledon was unaffected by the growth media sulfate salt concentration. This strongly suggests that the origin of the sinigrin in the *Brassica juncea* cotyledon is from the seed[77].

Milford et al., and Fieldsend and Milford studied the concentration of various glucosinolates in vegetative and floral tissues of *Brassica napus* cultivars[221,223]. High glucosinolate concentration was found to persist from the seed until the seedling stage. The glucosinolate concentration decreased as the seedling grew into the early vegetative stage. Then, the glucosinolate concentration increased in the late vegetative stage until floral tissues formed[221,223]. The general trend is for increased alkenyl-glucosinolate concentration with increased age in the vegetative tissues of *Brassica napus*[221,222,223].

For this thesis the sinigrin concentration in *Brassica juncea* leaf tissue has been presented. The glucosinolate concentrations of both Cutlass and Domo cultivars are similar (**Figures 18** and **19**). The decrease in the sinigrin concentration during the first few weeks of plant growth is consistent with the published work of Bodnaryk and Palaniswamy (*Brassica juncea* cv. Cutlass)[77]. The general trend of a decrease in the leaf sinigrin concentration until a minimum at approximately the third week of growth is

parallel to the literature describing *Brassica napus*[221,222,223,224]. After the initial decrease in leaf sinigrin concentration to a minimum an increasing sinigrin concentration phase was noted in the growing mature plant (**Figures 18** and **19**). The maximum concentration of sinigrin in *Brassica juncea* occurred near the end of the plant life cycle (approximately the seventh and/or eighth week after planting, **Figures 18** and **19**).

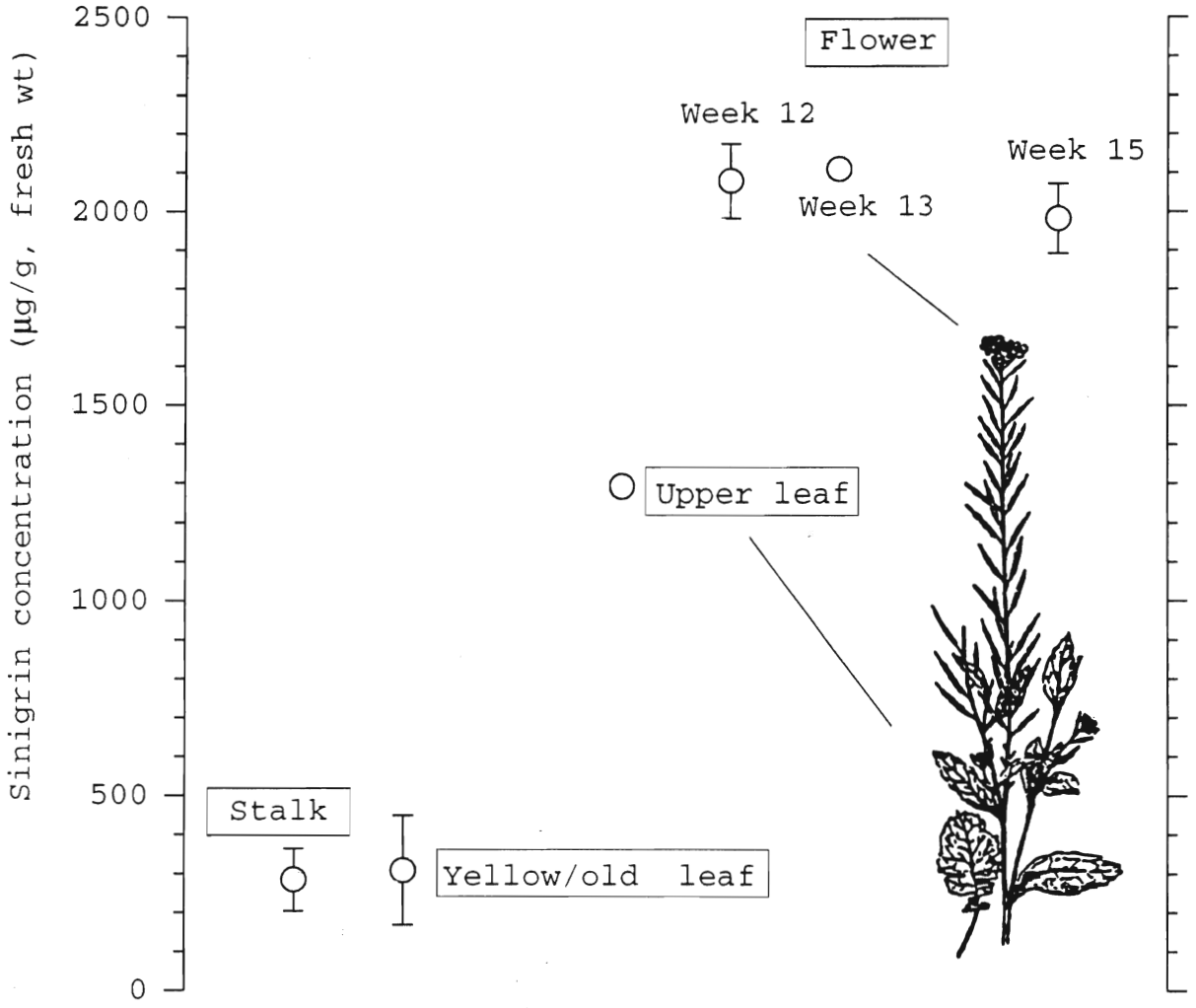
3) Comparison of different plant parts

The sinigrin concentrations of various *Brassica juncea* tissues (cultivars Cutlass and Domo) were studied. The results are presented in **Figures 20** and **21**. Plants were subdivided into tissue types for analysis. It was observed that two distinct leaf morphologies are present on the *Brassica juncea* plants. A thin blade-like leaf was produced during the flowering stage. The blade-like leaf only appeared on the upper portion the plant therefore it will be called an upper leaf.

A broad true leaf was produced after the second week of growth. The broad true leaf occurs on the lower portion of the plant and is called a lower leaf (**Figures 20** and **21**). Lower leaves, sampled at the eighth week after planting, are illustrated in **Figures 18** and **19**. Stalk was sampled from sturdy disease free sections. Yellow/old (senesced) leaves were still attached to the plant at the time of sampling.

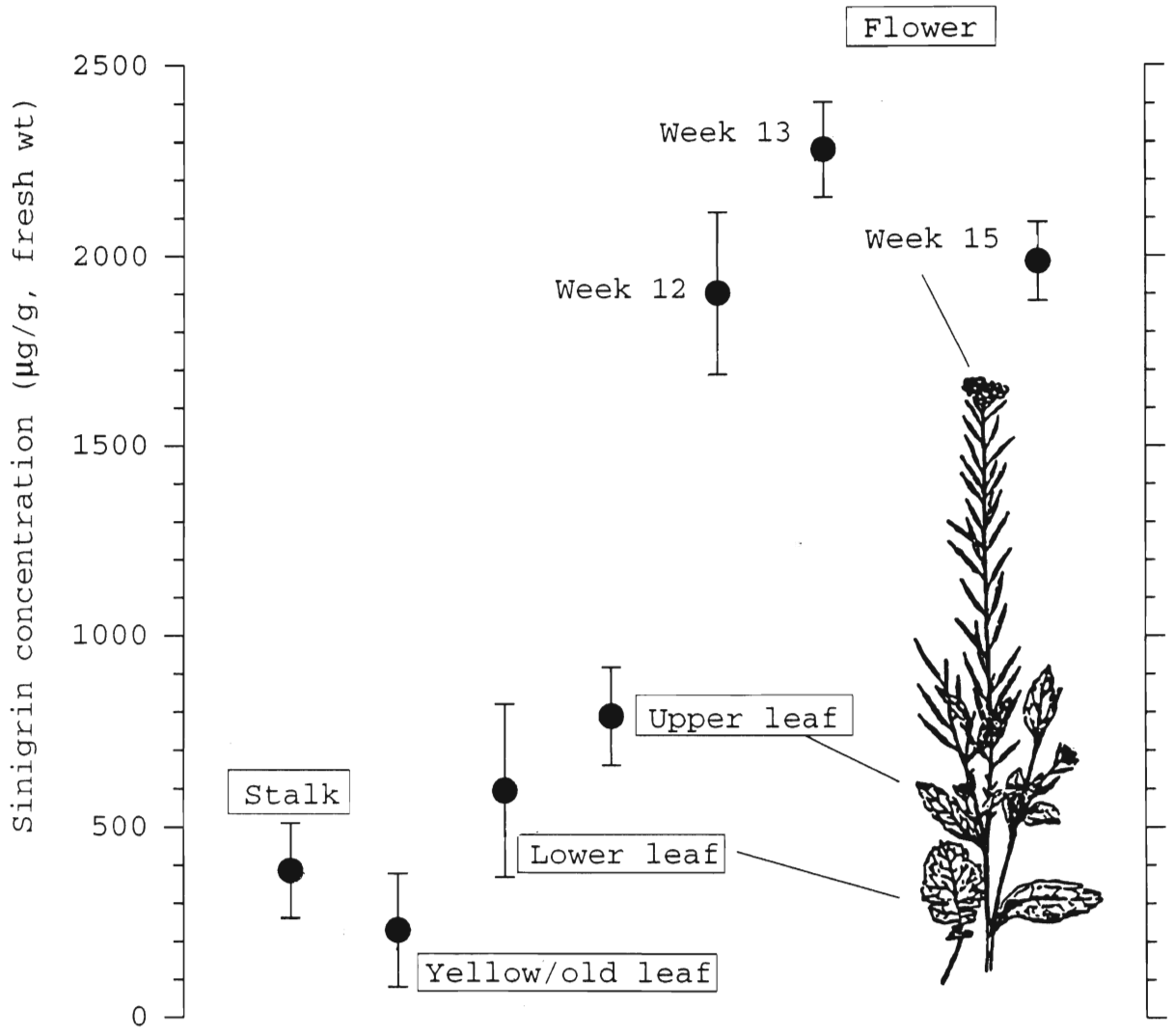
In this study, *Brassica juncea* flowers were the tissue containing the greatest concentration of sinigrin. *Brassica juncea* cv. Cutlass flowers contained a constant concentration of sinigrin at 2050$\pm$90µg/g over a three-week sampling period (**Figure 20**). The flowers of the Domo cultivar were observed to have a maximum sinigrin concentration of 2300$\pm$100µg/g (**Figure 21**). For both Cutlass and Domo cultivars, the stalk and yellow leaf samples contained a lower sinigrin concentration than the upper leaf. The concentration of sinigrin in the upper leaf was 1300µg/g (single

Figure 20: Comparison of the concentration of sinigrin in different parts of *Brassica juncea* cv. Cutlass



(All tissues were harvested at week 15, unless otherwise noted)

Figure 21: Comparison of the concentration of sinigrin in different parts of *Brassica juncea* cv. Domo



(All tissues were harvested at week 15, unles otherwise noted)

sample) and 800±100µg/g for Cutlass and Domo, respectively (**Figures 20** and **21**).

The sinigrin concentration of the stalk and lower leaf was similar for both cultivars. For Cutlass, the sinigrin concentration of the stalk was 280±80µg/g and the sinigrin concentration of the yellow leaf was 300±100µg/g (**Figure 20**). For Domo, the sinigrin concentration of the stalk was 400+100µg/g and the sinigrin concentration of the yellow leaf was 200+100µg/g (**Figure 21**). Furthermore, the concentration of sinigrin in the lower leaf was 600±200µg/g (**Figure 21**). It was observed that the greatest concentration of sinigrin in *Brassica juncea* occurred in the flowers (**Figures 20** and **21**). The roots were not sampled for this study because published reports suggested that sinigrin is not the major glucosinolate in root tissues[44].

Sang et al. studied the glucosinolate profile of various curciferous plants[44]. *Brassica juncea* cv. Zem-2 (mustard) was among the plants studied. Unfortunately, the glucosinolate concentrations of the selected plant tissues were not quantified. Sinigrin was found to be the predominant glucosinolate in the mustard leaf (traces of indolylmethyl-glucosinolates were also found)[44]. Mustard root was found to contain large amounts of 2-phenyl-ethyl-glucosinolate and some indolylmethyl-glucosinolates[44]. Analysis of *Brassica juncea* seed revealed predominantly sinigrin with traces of 4-hydroxy-3-indolylmethyl-glucosinolate[44].

The literature reveals that the glucosinolate concentration in *Brassica sp.* plants can be affected by cultivation techniques[220,225,226]. Glucosinolate levels increase with the application of sulfate salts[220,225] and decrease with the increasing application of nitrate salts[220]. Soil composition can also affect the concentration of glucosinolates in *Brassica sp.* plants[220]. Generally, plants grown in sandy soils have lower glucosinolate concentrations when compared to plants grown in heavy clay soils[220]. Furthermore, plant infection by certain pathogens may also increase

the level of glucosinolates in *Brassica napus* cultivars[226].

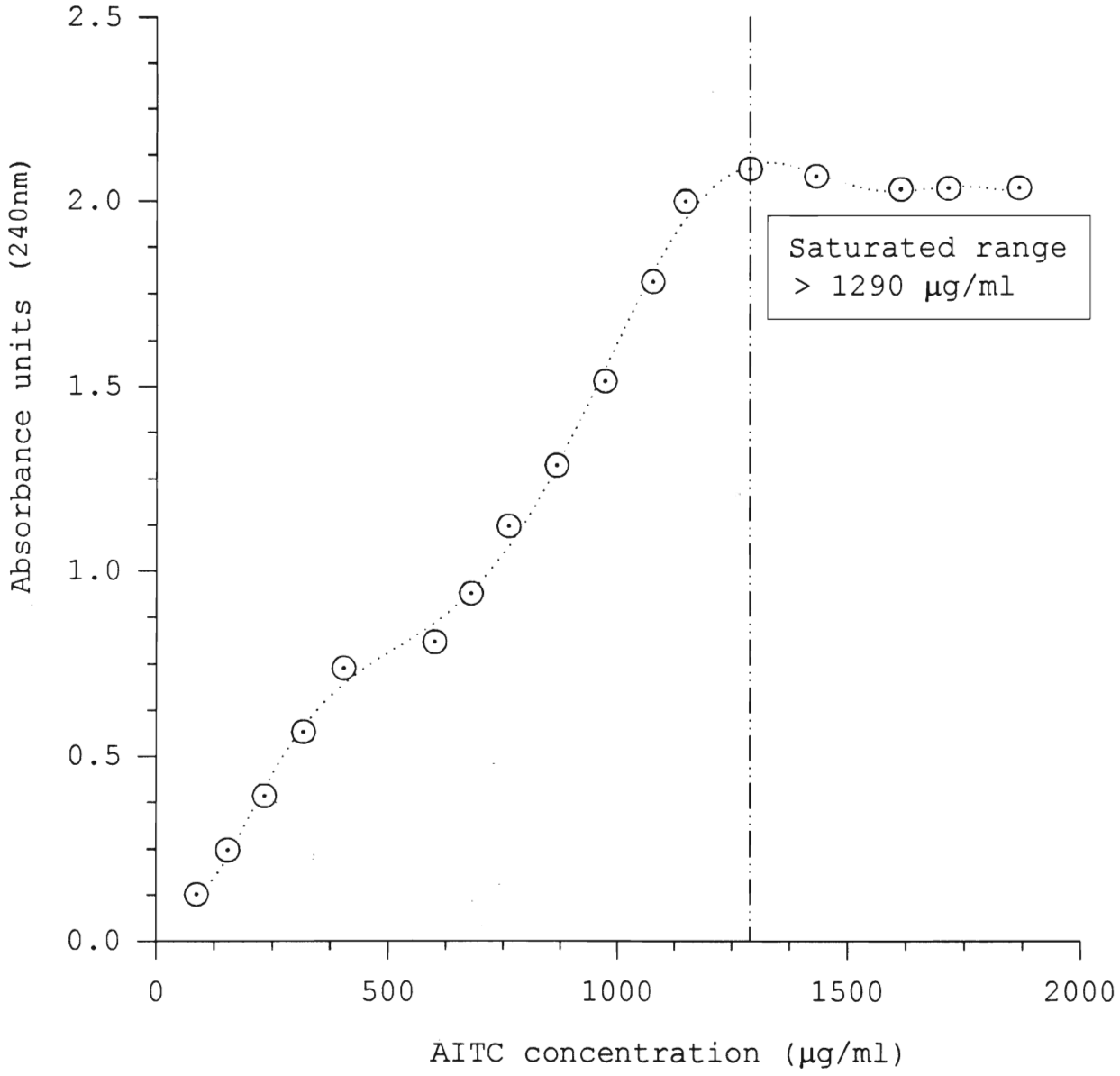
Judging from the data presented in this thesis the greatest concentration of sinigrin in *Brassica juncea* leaf tissue occurs at approximately five weeks after planting. In order to use *Brassica juncea* as a sustainable nematicide this stage may be the best time for harvest to incorporate the plant material into the green manure system. The greatest increase (approximately 200%) in the sinigrin concentration of the leaves occurred between the fourth and fifth week of growth (**Figures 18** and **19**). Although *Brassica juncea* flower tissues contain the greatest concentration of sinigrin (**Figures 20** and **21**), much of the plant mass at the flowering stage were yellow and dying leaves. Senesced leaves were shown to have a low sinigrin concentration (less than 500μg/g, **Figures 20** and **21**). For maximum yield of sinigrin, *Brassica juncea* plants should be harvested before the flowering stage. Further experiments are needed to determine the most productive stage to harvest plant for use as a sustainable nematicide.

7. Behavior of AITC

1) Solubility of AITC in water

For this thesis, the solubility of AITC in water was determined. The solubility was measured by determining the concentration at which a water solution became saturated with AITC. UV absorption was used to determine the level at which no more AITC would dissolve in water. Solutions were agitated for one hour, then 12 hours was allowed to elapse before analysis of the AITC concentration (see Experimental). The solubility of AITC in water was determined to be 1290μg/ml at approximately 24°C (**Figure 22**). AITC solubility determined for this thesis was near the maximum of the aqueous solution concentration range reported in the literature (178μg/ml[167] to 2000μg/ml[168]).

Figure 22: Solubility of AITC in water



2) Determination of AITC and degradation compounds by RP-HPLC

AITC has been reported to be unstable in aqueous solution[146,167,168,170]. For this thesis, a RP-HPLC-UV method was developed for the separation of compounds from a degraded aqueous AITC sample (see Experimental, **Figure 23** and **Table 3**). The goal of this study was to identify as many aqueous AITC degradation compounds as possible. However, the priority was to monitor the decrease in AITC concentration in the aqueous AITC samples with time.

Figure 23 illustrates the chromatogram of a 1030µg/ml aqueous (pH~7 phosphate buffer solution) AITC solution after approximately three months of storage at room temperature in the absence of light. The small insert chromatogram in **Figure 23** illustrates the sample after approximately four months of storage (retention times 22 to 35 minutes).

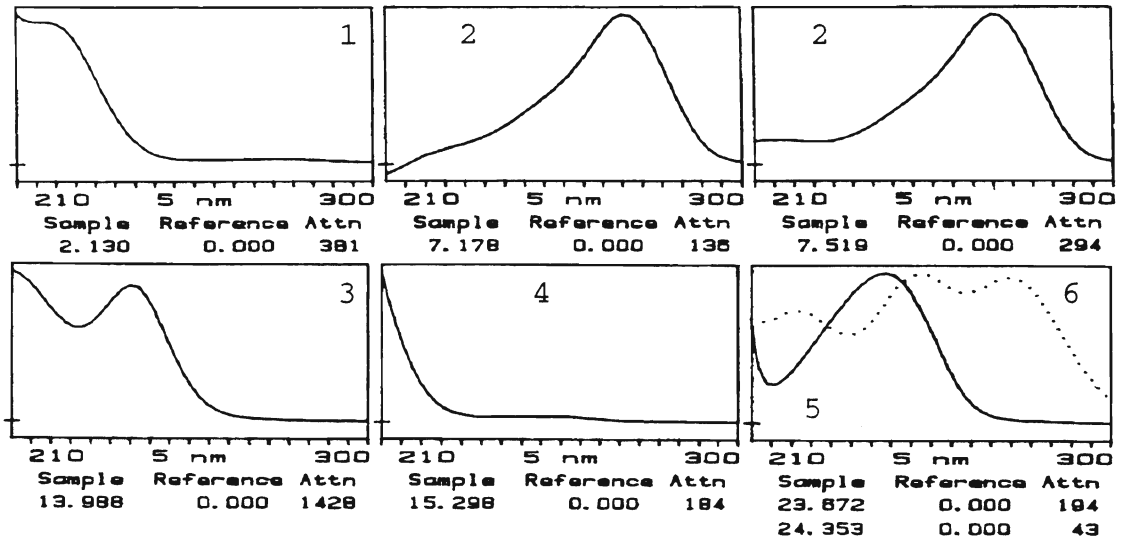
A mobile phase system consisting of acetonitrile/water was chosen for the determination of AITC (see Experimental and **Figure 23**). Terada et al. used an acetonitrile/buffer solution as the mobile phase for the determination of the related alkyl-isothiocyanate MITC[172]. This work presents the first report of an acetonitrile/water gradient mobile phase used for the determination of AITC. Furthermore, this is the first study in which the AITC aqueous degradation compounds were separated by a single analytical technique. Methanol was not used as the mobile phase organic modifier because alkyl-isothiocyanates react with methanol to produce a methyl-alkyl-thionocarbamate[147,167,170,177,178]. Significant degradation occurs when AITC standards are prepared in methanol[177]. Decomposition has been shown to occur over the course of a few days of sample storage[177].

The ultraviolet-visible absorption spectra of a series of alkyl-isothiocyanates have been studied in detail[190]. The predominant absorption band was found in the 244nm to 248nm region[190,227] though, greater UV absorbance occurs at approximately

Figure 23: Chromatogram of a degraded aqueous AITC sample

(for further details see Experimental, Results and Discussion)
for peak identification see **Table 3**

UV Spectra:



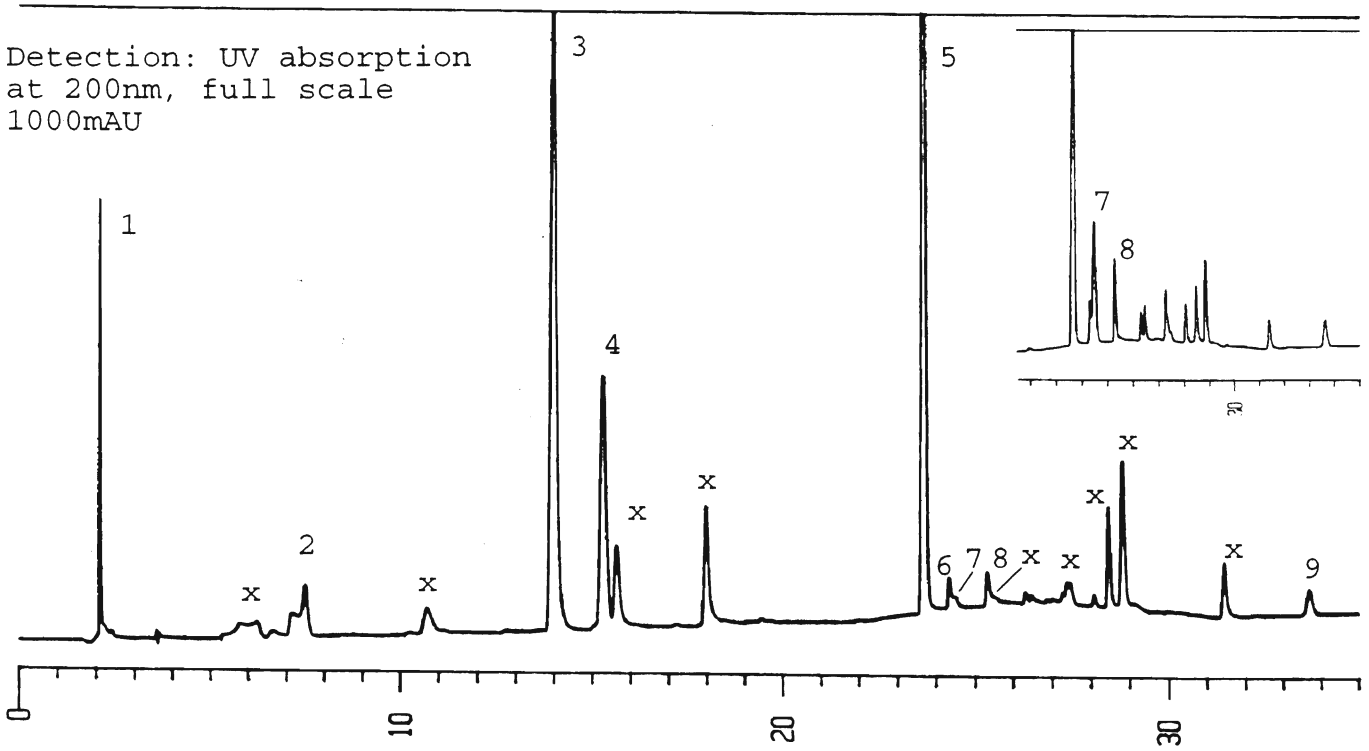


Table 3: Proposed identity of aqueous AITC degradation compounds separated by HPLC.

Identified peaks of the chromatogram illustrated in **Figure 23.** Please refer to Results and Discussion for further details about peak identification.

Peak #	Retention time (min)	Degradation compound
1	2.1	unknown (possibly allyl-thionocarbamic acid)
2	7.2, 7.5	allyl-thiourea (split peak)
3	14.0	1,3-diallyl-2-thiourea (DATU)
4	15.3	allyl-thiocyanate
5	23.7	allyl-isothiocyanate (AITC)
6	24.4	diallyl-dithiocarbamate (DAD)
7	24.5	diallyl-sulfide
8	25.3	diallyl-disulfide
9	33.7	unknown
x	~6.5, 10.7, 15.7, 18.0, 28.5, 28.9	mobile phase impurities

200nm[174,227]. Thiols and sulfides are potential AITC degradation products (see Introduction, **Figure 5**). Unfortunately, UV detection is expected to be not very sensitive for the detection of thiols or sulfides[227,228,229].

Commonly used HPLC grade solvents methanol, acetonitrile and water are essentially transparent in the alkyl-isothiocyanate UV absorption region (244nm to 248nm)[230]. Therefore, it is expected that UV detection should be suitable for the detection of alkyl-isothiocyanates separated by RP-HPLC. UV detection of thiols, sulfides, thiocyanates and other degradation compounds can be maximized by detection near 200nm. Acetonitrile has a UV absorption cutoff of less than 190nm compared with 205nm for methanol[231]. Seaver and Sadek recommend that acetonitrile should be used for gradient chromatography when low wavelength UV absorption detection is required[231].

Appendix 6, illustrates a blank chromatogram (200nm) for the RP-HPLC-UV method used to determine aqueous AITC (see Experimental). The retention times of the impurity peaks were stable (**Figure 23, Table 3** and **Appendix 6**). Peaks observed on the blank chromatogram (**Appendix 6**) were related to water quality and the peak areas varied with different mobile phase samples. Dead bacteria and bacterial growth by-products can cause ghost peaks with aqueous gradient chromatography[231]. For this study, the water portion of the mobile phase was changed daily to reduce the possibility of bacterial contamination.

i) Importance of acetonitrile in the sample

AITC decomposition studies have revealed that a range of degradation compounds are formed with widely varying properties[146,168,170] (see Introduction and **Figure 5**). Non-polar compounds are formed during the degradation of AITC in aqueous solution[168]. Precipitation of these compounds during the degradation process may occur. For this study, the reproducibility of AITC peak area was poor when the sample solution was directly injected into

the HPLC system as shown in **Table 4**. Also, a white precipitate was observed after as little as one week of degradation at room temperature.

Peak variability was reduced and total recovery of AITC was increased by amending the sample with acetonitrile before injection and HPLC analysis (**Figure 24** and **Table 4**). The sample solution was throughly shaken to suspend the precipitate. Then, the sample was mixed with acetonitrile to 50% (v/v) and AITC was determined by the RP-HPLC-UV method (see Experimental). Peak area reproducibility and total recovery were improved (**Figure 24** and **Table 4**). It is proposed that the AITC was adsorbed onto the non-polar precipitate. Acetonitrile resolubilizes the adsorbed AITC.

Increased recovery of AITC from the aqueous solution upon the addition of acetonitrile is illustrated in **Figure 24** and **Table 4**. The degradation of an aqueous AITC sample (pH=6.4, 310µg AITC/ml) was monitored (**Figure 24**). A lower AITC recovery was observed with the 100% aqueous sample. The AITC recovery of the 100% aqueous sample rapidly decreased with time (**Figure 24**). Increased recovery of AITC from the acetonitrile amended sample, compared with the 100% aqueous sample, was also noted (**Table 4**). The increased recovery from the acetonitrile amended sample was observed for samples between pH=5.7 and 8.6. Standard deviation of the peak area data was 7.5 to 33 times less for the sample that contained acetonitrile (50% v/v, **Table 4**).

ii) Identification of degradation compounds

Commercial AITC was used as an external standard for the determination of AITC by RP-HPLC-UV (see Experimental). AITC peak area measurements were made at 228nm. Blank chromatogram impurities (**Appendix 8**) were less prominent at 228nm than 200nm. The UV absorption detector response at 228nm was linear over the range of 2.3µg AITC/ml to 1140µg AITC/ml (**Appendix 6**).

Table 4: Comparison of the reproducibility of triplicate injections of aqueous AITC diluted with either buffer solution or acetonitrile

pH	Initial [AITC]†	aq. AITC sample★	Peak Area (mAUs)☆	Std. dev.
5.7	450	buffer	1500	± 300
5.7	450	ACN	2084	± 9
7.2	450	buffer	1500	± 200
7.2	450	ACN	1840	± 10
8.6	440	buffer	1200	± 300
8.6	440	ACN	1600	± 40

† µg/ml

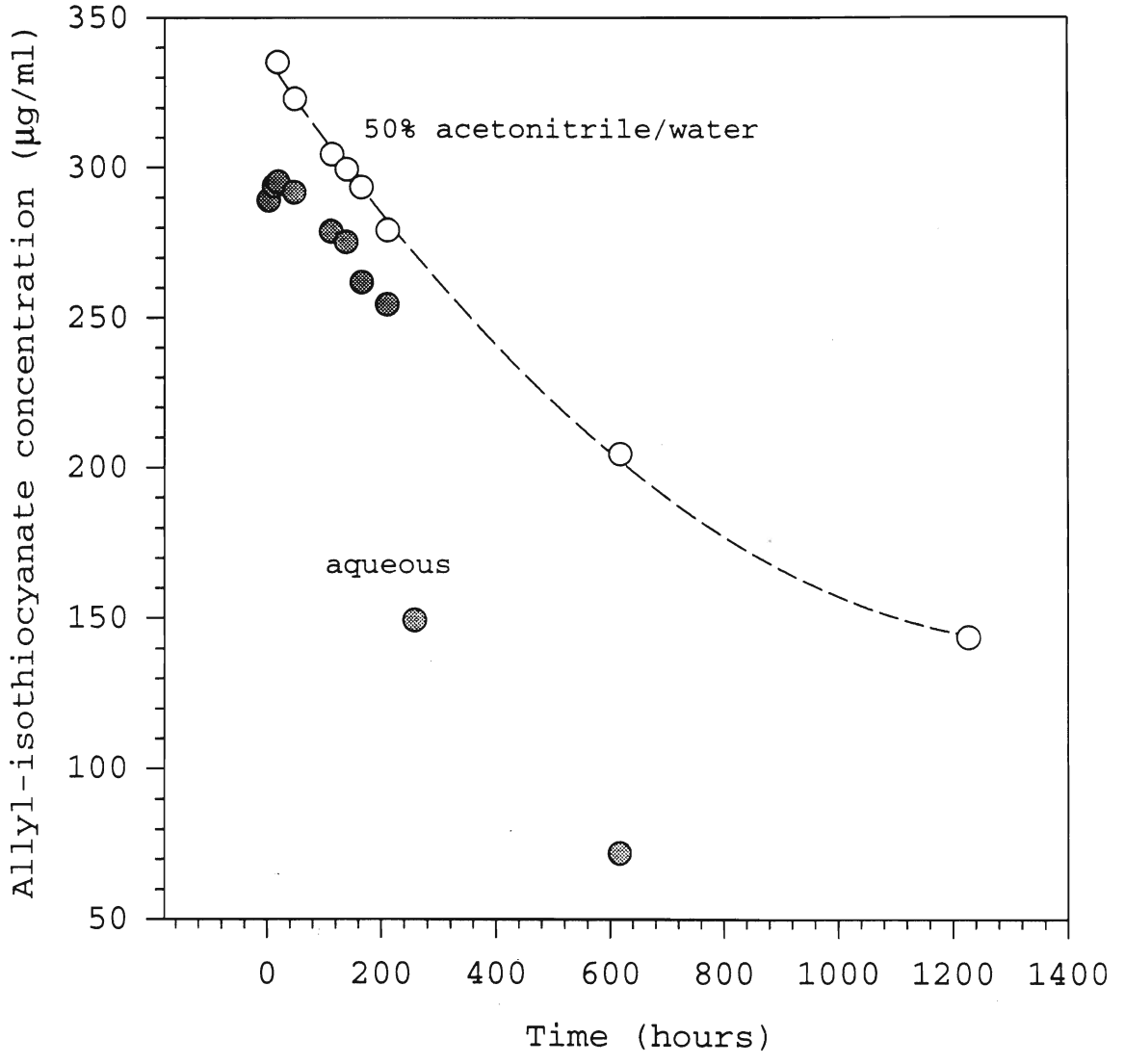
★ AITC sample (one week old) diluted with listed solution 50% (v/v)

ACN acetonitrile

buffer phosphate buffer solution at listed pH

☆ Peak area monitored at 240nm, AITC RP-HPLC-UV method was used (see Experimental)

Figure 24: Concentration of AITC in aqueous solution (25°C) versus time. Comparison of two methods of determination: injection of aqueous and 50% (v/v) acetonitrile/aqueous AITC samples



Vukmanic and Chiba studied the effect of organic solvent composition in the sample solution on the chromatographic peak profile[232]. Peak distortion and broadening may be observed when the acetonitrile concentration in the sample is higher than in the mobile phase[232]. In the current work, AITC retention time and peak width were only slightly influenced by the acetonitrile concentration in the sample. Less than 0.01 minute difference in retention time was noted between an AITC sample (25µl injection) prepared in 100% acetonitrile versus an aqueous sample. A similar effect of sample solvent composition upon peak width was also observed.

An attempt was made to locate and purchase standards of AITC degradation compounds as identified by Kawakishi and Namiki (**Figure 5**)[168]. Diallyl-thiourea (1,3-diallyl-2-thiourea, DATU) was located by an Aldrich Chemical Company computer search and purchased from the Sigma-Aldrich Library of Rare Chemicals. The purity of the purchased compound was unstated. The commercial standard was characterized by positive EI-MS (**Appendix 8**). The molecular ion ($M^{+\cdot}$, m/z=156) and an allyl-amine fragment ($[CH_2{=}CHCH_2NH]^+$, m/z=56) were observed in the mass spectrum (**Appendix 8**). The mass spectrum was consistent with that expected for a substituted thiourea[233]. Referring to **Figure 23,** DATU was identified as a major product of the aqueous degradation of AITC. Identification of the separated degradation compound was supported by UV absorption spectroscopy. The UV absorption spectrum (**Figure 23, peak #3**) was identical to a DATU reference spectrum[168].

Diallyl-sulfide and diallyl-disulfide were identified as AITC degradation compounds (**Figure 23** and **Table 3**). Diallyl-sulfides were identified by comparison of the retention times and UV spectra with commercial standards. The UV absorption spectra of diallyl-sulfide and diallyl-disulfide are illustrated in **Appendix 8**. The insert chromatogram on **Figure 23** revealed that diallyl-sulfide, diallyl-disulfide and other unknown non-polar compounds accumulate with time during AITC degradation in aqueous solution.

Diallyl-sulfide and higher sulfides may form from thiols as proposed by Kawakishi and Namiki[168]. Thiols readily undergo oxidative coupling to give dialkyl-sulfides[234]. The formation may occur by air, halogen, hydrogen peroxide or Fe^{3+} oxidation[234]. Kawakishi et al.[146] and Kawakishi and Namiki[168] reported that AITC degraded to diallyl-disulfide but not diallyl-sulfide. In my research, both compounds were observed as degradation products (**Figure 23** and **Table 3**). Possibly, diallyl-sulfide was oxidized to diallyl-disulfide during the isolation in their studies[146,168].

Diallyl-dithiocarbamate (DAD) has been suggested as a probable intermediate in the aqueous degradation of AITC (**Figure 5**)[168]. Drobnica and Gemeiner studied the decomposition of alkyl-dithiocarbamate esters in aqueous solution[235]. In aqueous solution alkyl-dithiocarbamate esters form an equilibrium mixture with an alkyl-thiol and alkyl-isothiocyanate[235]. Degradation of the alkyl-isothiocyanate was found to proceed to an alkyl-thionocarbamate (RNHCSOH)[235]. The stability of alkyl-dithiocarbamate esters is reported to decrease in alkaline solution[235].

For this thesis, DAD was synthesized from sodium allyl-mercaptide and AITC. The product was purified by a preparative HPLC procedure (see Experimental). A reversed-phase analytical column was overloaded and the heart of the DAD peak was collected. Purified DAD was collected as a 30% acetonitrile/water (v/v) solution.

Identity of the ester was confirmed by mass spectrometry and UV absorption (**Figure 23** and **Appendix 8**). The UV absorption spectrum of the ester (**Figure 23** and **Appendix 8**) was identical to a reference spectrum[168]. The PB (EI) mass spectrum (**Appendix 8**) was similar to a published EI spectrum[168] and consistent with established fragmentation schemes[236,237]. Important fragments observed in the HPLC-PB-EI mass spectrum were: $[MH]^+$ m/z=174 and $[CH_2{=}CHCH_2N{=}C{=}S]^+$ m/z=99 (**Appendix 8**). In this study, DAD was identified as an aqueous degradation product of AITC (**Figure 23, peak #6**).

In an attempt to identify the early eluting degradation components, sodium-allyl-dithiocarbamate (SAD) was synthesized and purified by established procedures[238,239]. SAD displayed a UV absorption maximum at 218nm (**Appendix 8**). SAD was found to be eluted at the system peak coincident with the retention time of **peak #1** (**Figure 23** and **Table 3**). **Peak #1** displayed a UV absorption spectrum that was similar to SAD (**Figure 23**). The mono-oxygen analog of SAD, sodium-allyl-thionocarbamate, was the expected aqueous AITC degradation compound[168]. Sodium-allyl-thionocarbamate is characterized by a single strong UV absorption band at 227nm[190]. Kawakishi and Namiki have reported that allyl-thionocarbamic acid decomposes into allyl-amine in aqueous solution[168]. Similarly, Lapatechi and Newton studied the degradation of alkyl-dithiocarbamic acids in aqueous solution[240]. Degradation in aqueous solution proceeds to the alkyl-amine[240]. Also, stability decreases as the reaction mixture is acidified[240].

During my research, allyl-thiocyanate was discovered as a degradation product of AITC prepared in 100% acetonitrile solution. AITC in acetonitrile was observed to degrade into a single compound as assayed by the RP-HPLC-UV method for AITC determination (see Experimental). The isomerization of AITC into allyl-thiocyanate has been established in the literature[58,241]. AITC isomerization has been reported to be facile[58,241] and roughly independent of the solvent polarity[58].

The identity of allyl-thiocyanate in the AITC acetonitrile standard was confirmed by GC-MS and UV absorption spectroscopy (**Appendix 8**). Alkyl-isothiocyanates and alkyl-thiocyanates display contrasting mass spectra (see Introduction and **Appendix 8**). The featureless UV absorption spectra (**Figure 23, spectra #4** and **Appendix 8**) is consistent with an alkyl-thiocyanate[227]. For this study, allyl-thiocyanate was observed during the aqueous degradation of AITC (**Figure 23** and **Table 3**).

For the current work, allyl-thiourea was identified as an aqueous AITC degradation product by comparison with a commercial

standard. In **Figure 23**, the double peak (**peak #2**) was identified as allyl-thiourea. The presence of acetonitrile in the ammended sample caused the early eluting allyl-thiourea peak to be split. Allyl-thiourea was not reported by Kawakishi and Namiki as a degradation product of AITC in aqueous solution (**Figure 5**)[168]. It is known that alkyl-isothiocyanate reacts with ammonia to form an alkyl-thiourea[242]. Possibly, allyl-thiourea may form from AITC and ammonia present in the aqueous degradation solution.

Aqueous AITC samples prepared in pH=4.97, 6.52 and 9.07 phosphate buffer solutions and stored at 35°C for one week were analyzed by RP-HPLC-UV (**Figure 25**). Comparison of degradation at the three pH values revealed that the decomposition pathway to DATU (proposed by Kawakishi and Namiki[168]) was favored at basic pH (**Figure 25**). Under acidic conditions degradation of AITC in aqueous solution into diallyl-sulfide was favored (**Figure 25**). Referring to the sample (pH=6.3) monitored in **Figure 27**, after 2762 hours of degradation at room temperature 39.1% of the initial AITC had degraded into DATU and 14.7% of the initial AITC remained.

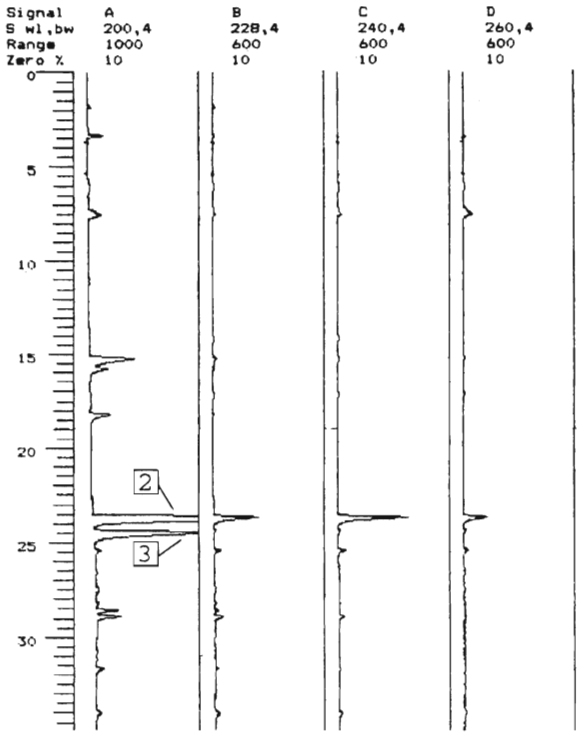
Presented in **Figure 26** is a scheme for the degradation of AITC in aqueous solution. The scheme (**Figure 26**) is based upon the pathway proposed by Kawakishi and Namiki (**Figure 5**)[168]. Incorporated in **Figure 26** are the AITC aqueous degradation compounds discovered in the present study: allyl-thiourea, allyl-thiocyanate and diallyl-sulfide. Furthermore, the results of this thesis suggest that aqueous AITC degradation into DATU is favored at basic pH and degradation into diallyl-sulfide is favored at acidic pH.

iii) Rate of degradation

The decrease in the AITC concentration with time for an aqueous AITC solution at 25°C was monitored. **Figure 27** illustrates the decrease in AITC concentration with time for aqueous AITC samples at two initial AITC concentrations. A curve was fit to the data in **Figure 27**. Half-lives of AITC decomposition (room temperature) were calculated directly from the curve. An initial

Figure 25: Degradation of AITC in aqueous solution at 35°C (pH=4.97, 6.52 and 9.07)

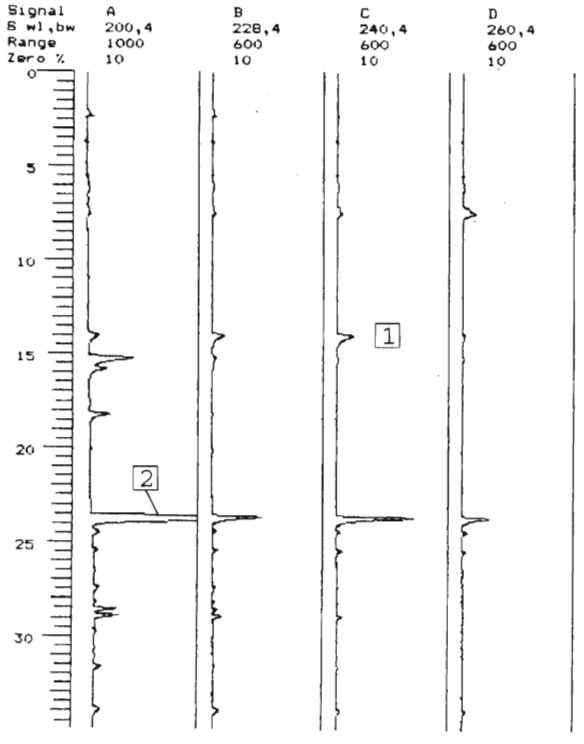
1010 µg/ml AITC, pH=4.97



(for further details see Experimental, Results and Discussion)

1= 1,3-diallyl-2-thiourea
2= allyl-isothiocyanate
3= diallyl-sulfide

1006 µg/ml AITC, pH=6.52



1001 µg/ml AITC, pH=9.07

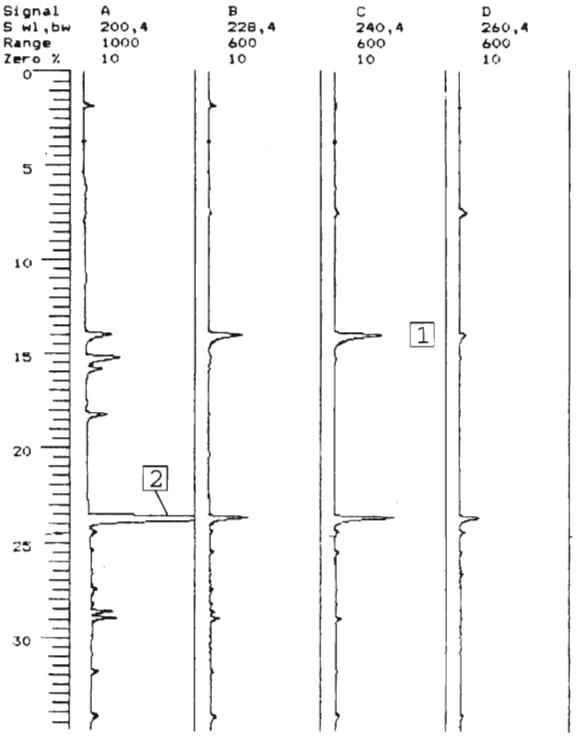


Figure 26: Degradation of allyl-isothiocyanate in aqueous solution (revised scheme)

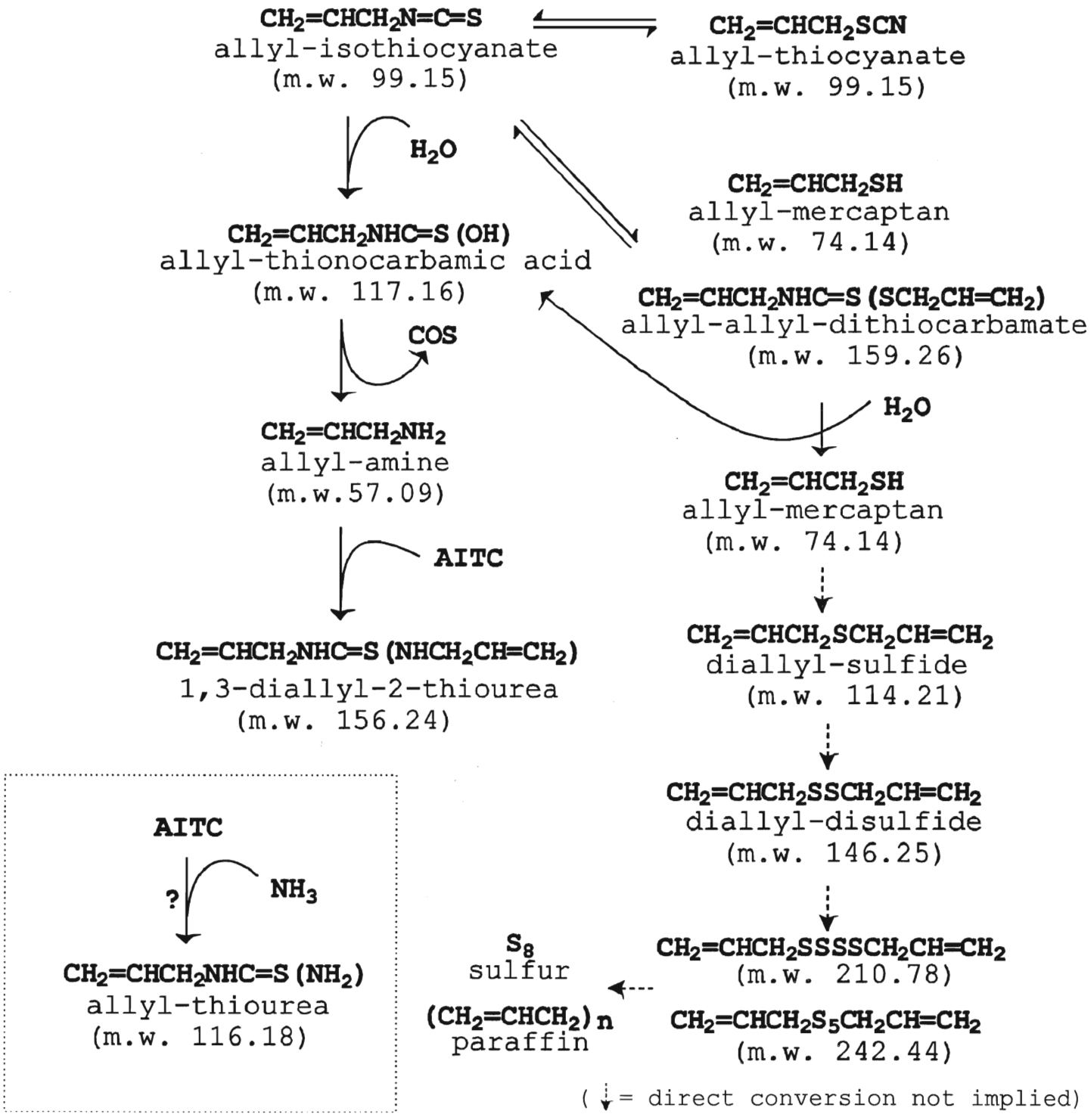
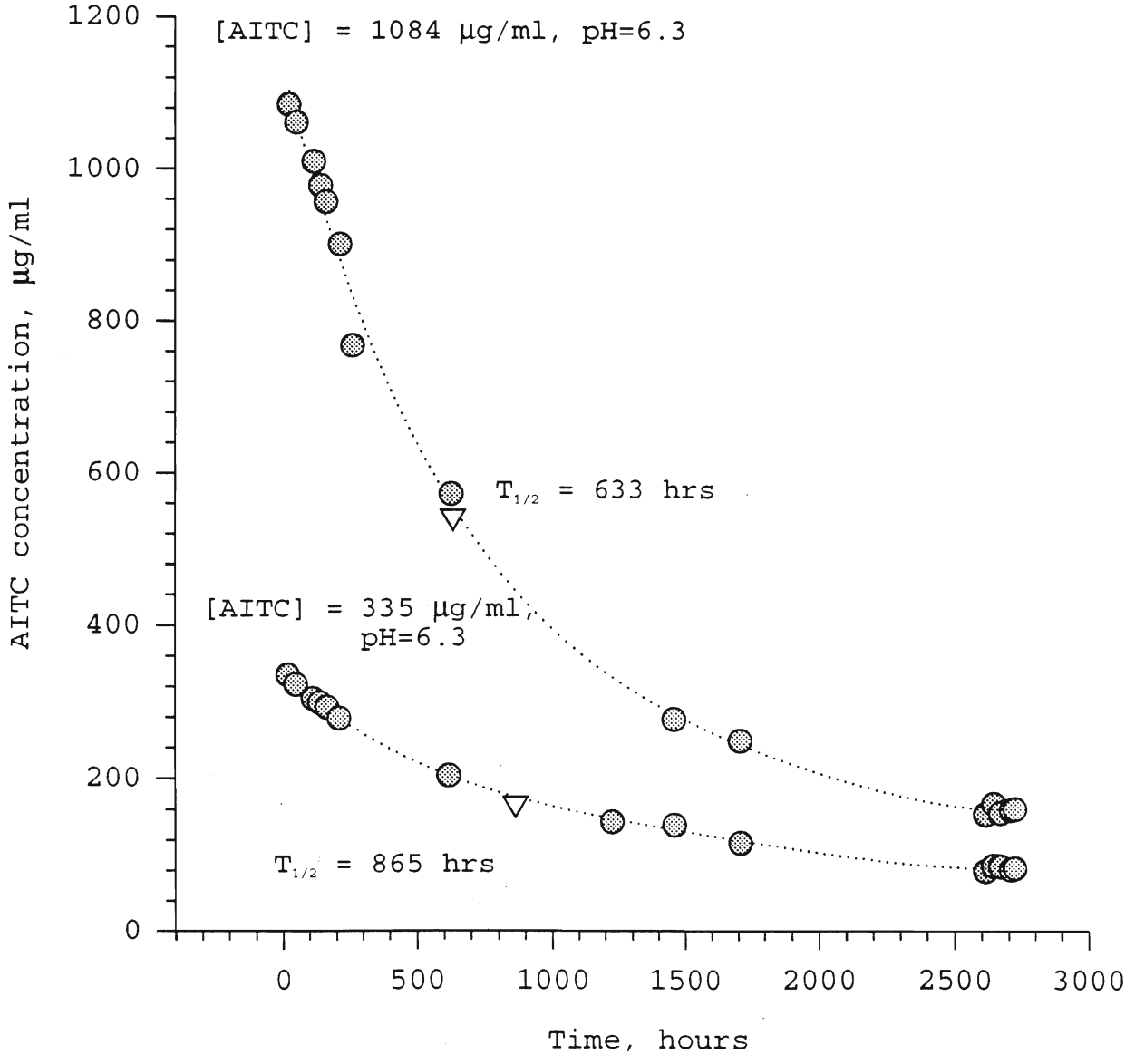


Figure 27: Degradation of AITC in aqueous solution at 25°C



AITC concentration of 1084µg/ml had a half-life of 633 hours at 25°C in pH=6.3 phosphate buffer solution (**Figure 27**). The AITC half-life for an aqueous solution (pH=6.3 phosphate buffer solution) with an initial AITC concentration of 335µg/ml was 865 hours at 25°C (**Figure 27**).

For this thesis, the rate of AITC degradation at 35°C was unaffected by the pH of the solution over the range of pH=4.97 to 9.07 (**Figure 28**). The half-life of AITC (pH=4.97 to 9.07 and initial concentration of 1000µg/ml) was determined to be 76±4 hours at 35°C (**Figure 28**). The results of this investigation are similar to that reported by Kawakishi and Namiki[168]. Analysis of the AITC degradation data provided by Kawakishi and Namiki revealed a half-life in aqueous solution of approximately 4.5 days (initial concentration 400µg/ml, pH=5.2 and 37°C)[168]. Based upon the AITC degradation data (**Figure 27** and **Figure 28**), the loss of AITC from aqueous solution was not adequately described by first or second order kinetics.

3) Degradation of AITC in soil

For this thesis, the degradation of AITC in soil at 35°C was studied. The soil was amended with AITC to give an initial concentration of 440±60µg/ml in the soil moisture. AITC was added at a concentration less than the aqueous solubility of AITC (1290µg/ml, see Results and Discussion). Extraction of the soil involved the use of water. A chromatogram of an aqueous extract of AITC amended soil is shown in **Figure 29.** It can be observed that aqueous coextractives from the soil did not interfere with the determination of AITC (blank chromatogram, **Figure 29**).

Many other researchers have used organic solvents for the recovery of alkyl-isothiocyanates from soil[45,153-156,161,163]. For the current study, an attempt was made to determine AITC in the aqueous phase of the soil media. Aqueous phase AITC may be related to the AITC available for nematicidal action. Sood and Sood proposed that

Figure 28: Degradation of AITC in aqueous solution and soil at 35°C

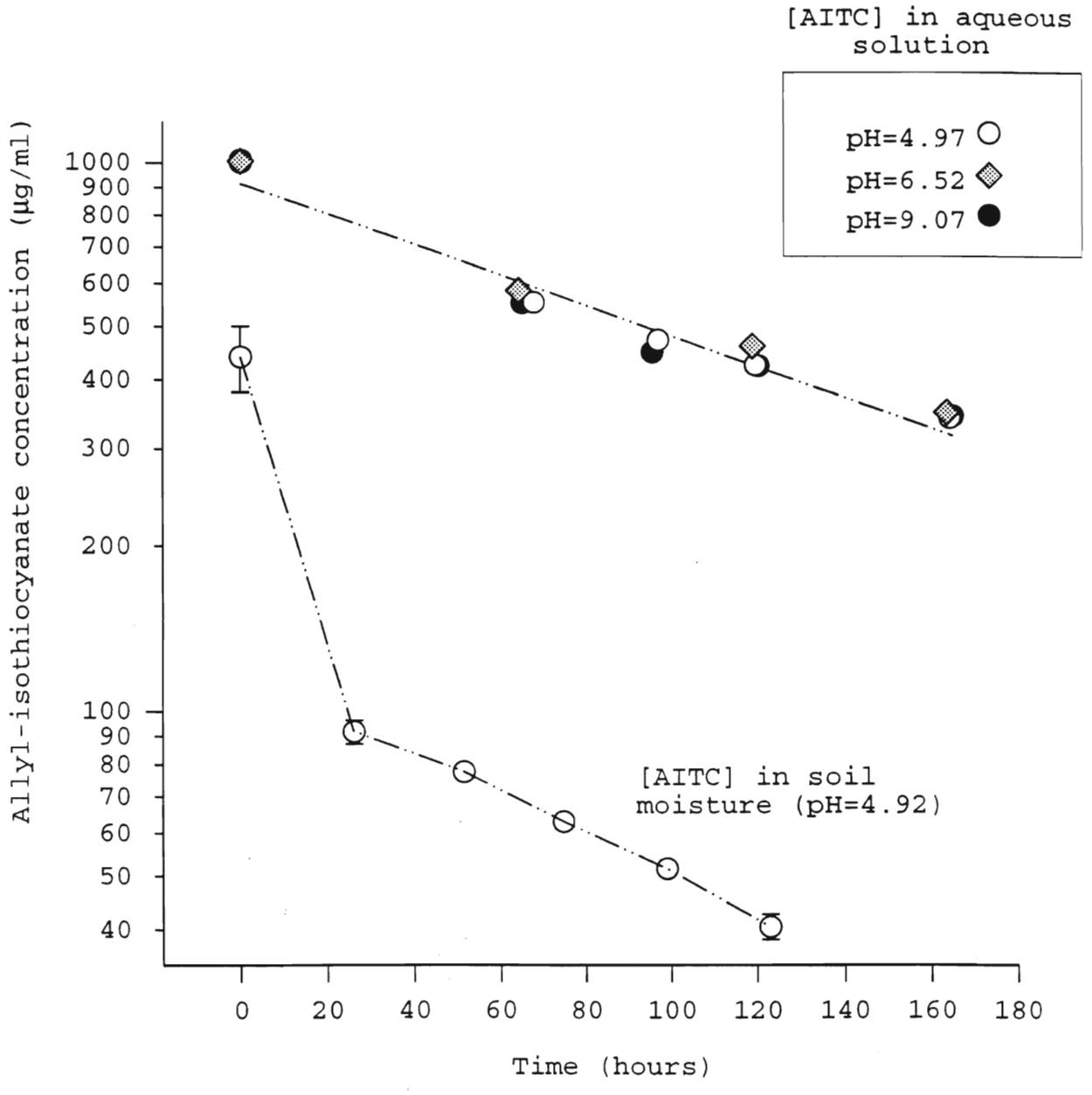
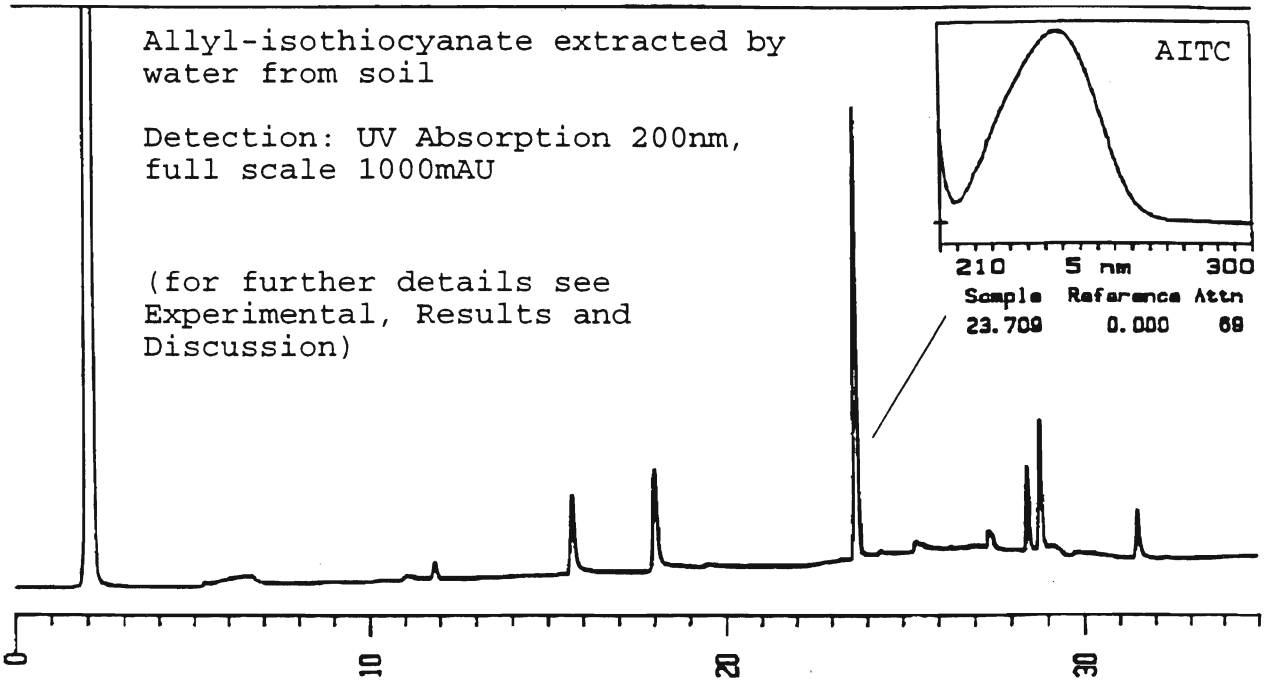
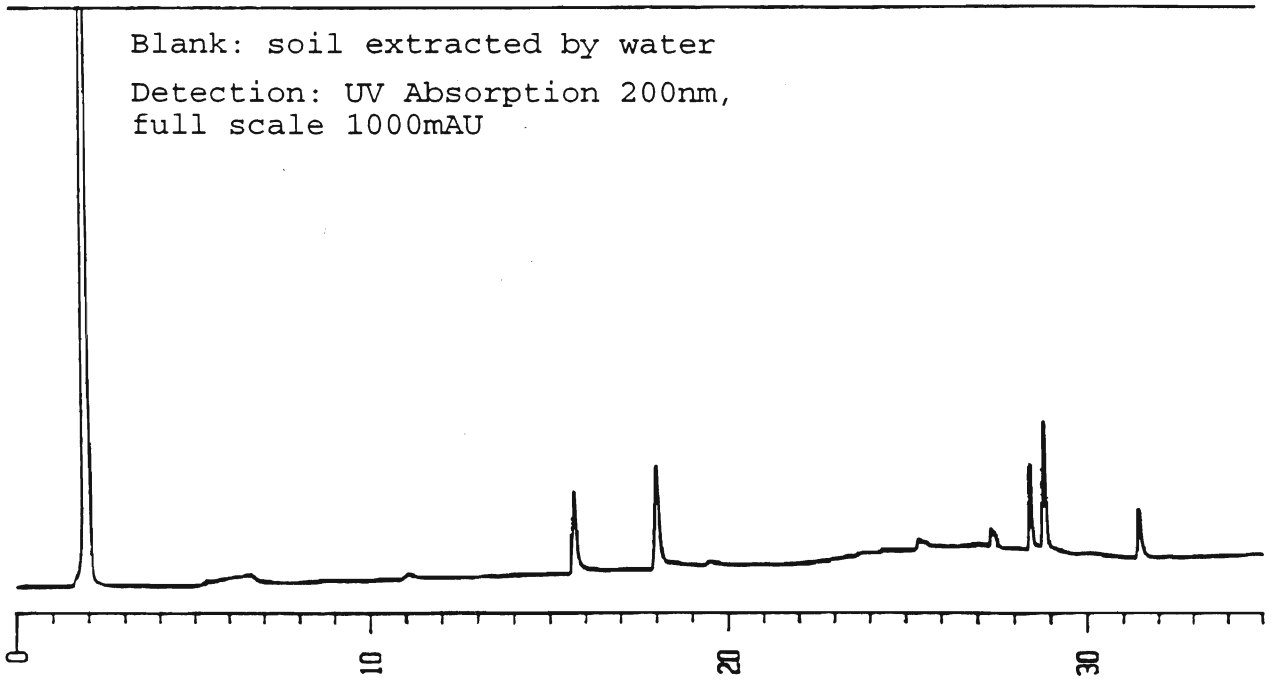


Figure 29: Chromatogram of AITC extracted from soil by water





solutes must diffuse across the outer membrane of the nematode from a surrounding water film[169]. Considering the work of Sood and Sood[169], experiments should be performed to determine if the nematicidial action of AITC in soil is adequately described by the aqueous phase component alone.

A substantial (75%) reduction in the AITC concentration was observed within 24 hours after amendment of the soil sample (**Figure 28**), possibly as result of adsorption in the soil. Adsorption in soil is an important factor affecting the mobility and availability of MITC for nematicadal action[158]. After the large initial loss of AITC, however, degradation in soil was parallel to degradation in aqueous solution (**Figure 28**). The exact nature of the initial loss of AITC needs to be studied by further experiments.

4) Toxicological aspects of AITC

The toxicity of aqueous AITC solutions to the root-lesion nematode (*Pratylenchus penetrans*) was determined (see Experimental). The study was completed as a collaborative effort with Mitch Pagoda and Dr. Potter (Agriculture and Agri-Food Canada). A plot of nematode mortality versus AITC concentration is illustrated in **Appendix 9**. The EC_{50} of aqueous AITC against the root-lesion nematode was determined to be approximately 20µg/ml when the nematode was exposed to the test solution for one hour.

Further toxicology studies were conducted with a myrosinase treated *Brassica juncea* extract (see Experimental). Myrosinase treatment of the *Brassica juncea* extract afforded nearly quantitative conversion of sinigrin into AITC (see Experimental). Nematode mortality from the use of the myrosinase treated *Brassica juncea* extract is included in **Appendix 9**. The myrosinase treated *Brassica juncea* extract was approximately the same efficacy as an aqueous AITC solution of equivalent concentration. Therefore, the nematicidal action of myrosinase treated *Brassica juncea* extracts can be reasonably modeled by aqueous AITC solution.

CONCLUSION

Nematodes cause great economic losses in many crops[2-6,8]. Control of soil nematode infestation is heavily dependent upon the use of soil fumigation. Often soil fumigants are highly toxic and must be applied by specially trained contractors[10]. Furthermore, soil fumigation is expensive and a low cost alternative would allow nematode control measures to be applied to more crops[2].

The goal of this thesis was to study factors related to the development of *Brassica juncea* as a sustainable nematicide. *Brassica juncea* was selected as a low cost natural nematicide characterized by the glucosinolate sinigrin[23,42,43]. Sinigrin is the precursor of the strong nematicidal agent AITC[50,51]. In order to more clearly define the optimal utilization of *Brassica juncea* this thesis was focused upon two main areas. Firstly, methods were developed to determine the distribution of sinigrin in *Brassica juncea* cultivars Cutlass and Domo. Also, sinigrin concentrations in plant tissues at various stages of growth were determined. Secondly, the physical properties of AITC were studied and a method was developed for AITC determination.

Extraction was found to be a critical step for the determination of sinigrin in *Brassica juncea* tissues. A diffusion extraction[77,80,85] method was developed to extract sinigrin from whole *Brassica juncea* tissues. Plant tissues were left intact and boiling solvent was used during the extraction procedure to minimize sinigrin losses. The optimal extraction time with either phosphate buffer or methanol/water boiling solutions was 25 minutes. Methanol/water extracted 13% greater amount of sinigrin than a phosphate buffer solution. Greater extraction efficiency

of the aqueous methanol solvent suggests the presence of a stress-induced waxy layer on the *Brassica juncea* leaves[199]. Phosphate buffer solution was selected as the extraction solvent for routine analysis because of greater compatibility with the determination procedures.

The stability of sinigrin was determined under the conditions of the diffusion extraction procedure. The study was initiated because of known variability of glucosinolate stability in different solvent systems[39,43,200,201]. The low rate of sinigrin degradation reported in this thesis is consistent with published accounts[39,40,41]. Experimentally, during the 25 minute diffusion extraction procedure only 3.7% degradation of sinigrin occurs.

Brassica juncea extract clean up was accomplished by an ion-pair SPE method. This method was developed independently of a recently published report by Betz and Fox[78]. The recovery of sinigrin was 92.6% and coextractive impurities were not detected in the cleaned up extract. The method developed for this thesis is a substantially better than that of Betz and Fox[78].

Various RP-HPLC-UV based methods were developed for the determination of sinigrin in the aqueous extract. An isocratic mobile phase separation utilizing ammonium acetate solution was developed. The separation was convenient for the determination of sinigrin in aqueous *Brassica juncea* extracts. Extracts were directly injected into the HPLC system without additional clean up. An isocratic ion-pair mobile phase system was also developed for the RP-HPLC-UV determination of sinigrin. An advantage of the method was the direct determination of sinigrin in 70% methanol/water *Brassica juncea* extracts without further clean up. A disadvantage of the method was the observed harmful effect of

the ion-pair reagent upon the chemically-bonded silica analytical column.

A novel step gradient system was also developed for the RP-HPLC-UV determination of sinigrin. The method involved preconditioning the analytical column with phosphate buffer solution and then switching the mobile phase to 100% water after sample injection. A desirable aspect of this method was that the phosphate buffer salts are washed from the system during each analysis cycle.

Sinigrin and benzyl-glucosinolate were both determined by HPLC-PB-NCI-MS. Comparison of the mass spectra revealed the presence of similar fragmentation patterns. Fragments arising from the thioglucose moiety and side-chain were observed in both spectra. Potentially, this method may be developed for glucosinolate structure elucidation.

Sinigrin concentration in *Brassica juncea* cultivars Cutlass and Domo was studied. Both cultivars showed similar concentrations of sinigrin during the plant growth and in various plant parts. For Cutlass, the minimum and maximum sinigrin concentrations in the top three leaves were observed at the third and seventh week after planting respectively. For Domo, the minimum and maximum concentrations were observed at the fourth and eighth week after planting respectively. For both cultivars, the highest sinigrin concentration was observed in floral tissues and the concentrations in senesced leaf and stalk tissues were low.

Physical properties of the nematicidal agent AITC were determined. The solubility of AITC in water was determined to be

approximately 1290µg/ml at 24°C. In a collaborative effort with Mitch Pogoda and Dr. Potter (Agriculture and Agri-Food Canada), the nematicidal action of AITC was quantified. The EC_{50} of aqueous AITC against the root-lesion nematode (*Pratylenchus penetrans*) was determined to be approximately 20µg/ml at one hour exposure. It should be noted that EC_{50} values can be expected to be lower for longer exposures. The full AITC exposure response profile should be determined.

Degradation of AITC in water was studied and compared with data reported previously[146,167,168,170]. A RP-HPLC-UV method was developed for the separation of degradation compounds from aqueous AITC sample solutions. The method is a considerable improvement over the cumbersome multi-step procedure previously reported[146,168]. It was necessary to amend the aqueous AITC sample solutions with acetonitrile before injection into the HPLC system. The acetonitrile amendment considerably improved AITC recovery and reproducibility of the results.

For this investigation, the rate of AITC degradation in aqueous solution was determined. The half-life of AITC in water at 25°C did not follow integer-order kinetics. Beginning with a 1084µg/ml solution, the half-life was 633 hours. At 35°C the half-life AITC was 76 hours essentially independent of solution pH. Some degradation compounds identified have not been reported in the literature: allyl-thiourea, allyl-thiocyanate and diallyl-sulfide. Degradation to diallyl-thiourea was favored at basic pH and degradation to diallyl-sulfide was favored at acidic pH.

AITC degradation was also studied in soil at 35°C; after 24 hours approximately 75% of the initial AITC addition was unrecoverable by water extraction. Following the large initial

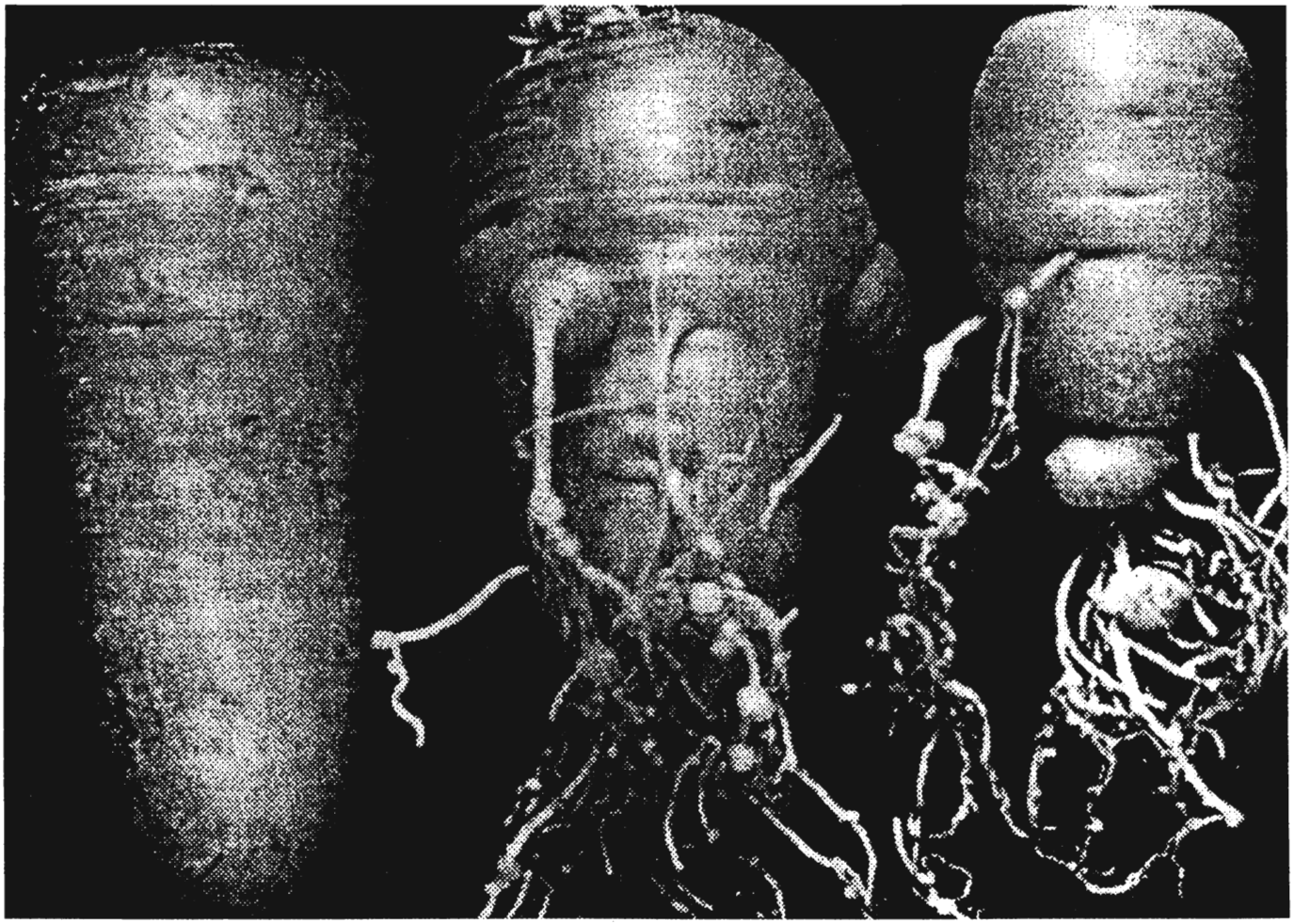
decrease in sinigrin concentration, the rate of degradation in soil was parallel to that in aqueous solution. The initial large loss of AITC should be further studied to determine if AITC was adsorbed in the soil media and is unavailable for nematicidal action.

Overall, *Brassica juncea* tissues are characterized by a high concentration of sinigrin. Sinigrin concentration generally increases with age of the plant. Judging from the data presented in this thesis the greatest concentration of sinigrin in *Brassica juncea* leaf tissue occurs after approximately five weeks of plant growth. In order to use *Brassica juncea* as a sustainable nematicide this stage may be the best time for harvest to incorporate the plant material into the green manure system. Although, the best harvest date will depend upon the growing conditions.

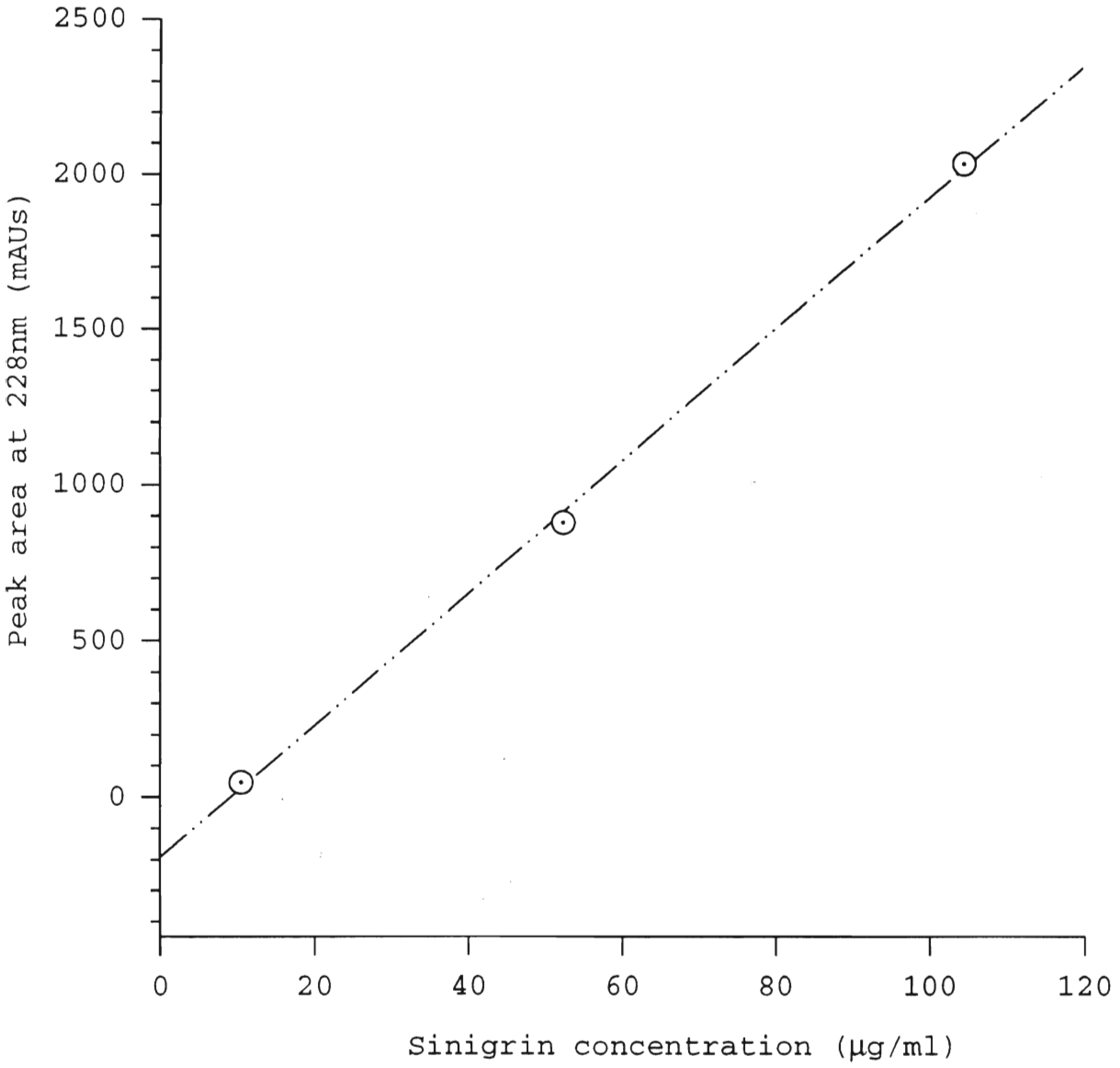
Preliminary work revealed that the enzymatic (myrosinase) conversion of sinigrin into AITC is quantitative. The lowest concentration of sinigrin observed in *Brassica juncea* leaf tissue was approximately 200μg/g (fresh weight). Quantitative conversion of singrin into AITC would give a concentration of approximately 55μg/g. For use of the plant as a nematicide, plant material and soil are mixed and enzymatically formed AITC is released into the aqueous phase of the soil (approximately 13% moisture content, see **Experimental**). If plant material and soil are mixed in a 1:1 weight ratio, then the aqueous phase AITC concentration will exceed the one hour EC_{50} value of AITC against the root-lesion nematode. The effect of AITC sorption in the soil must be considered in more detailed theoretical calculations.

Clearly, *Brassica juncea* has the potential to be developed into a low cost sustainable nematicide. Many necessary analytical procedures were developed in this report that will allow for further development. Specifically, HPLC methods were developed to allow for the rapid determination of sinigrin. Extraction was noted to be a critical step for the determination of sinigrin in *Brassica juncea* tissues. Future work should be directed towards determining tissue degradation and the release of AITC in soil. Also, the EC_{50} value of AITC against the root-lesion nematode should be determined in soil. This thesis presents a broad range of experimental procedures that have supported an investigation into the use of *Brassica juncea* as a sustainable nematicide. The procedures developed in the report will be useful for future investigations.

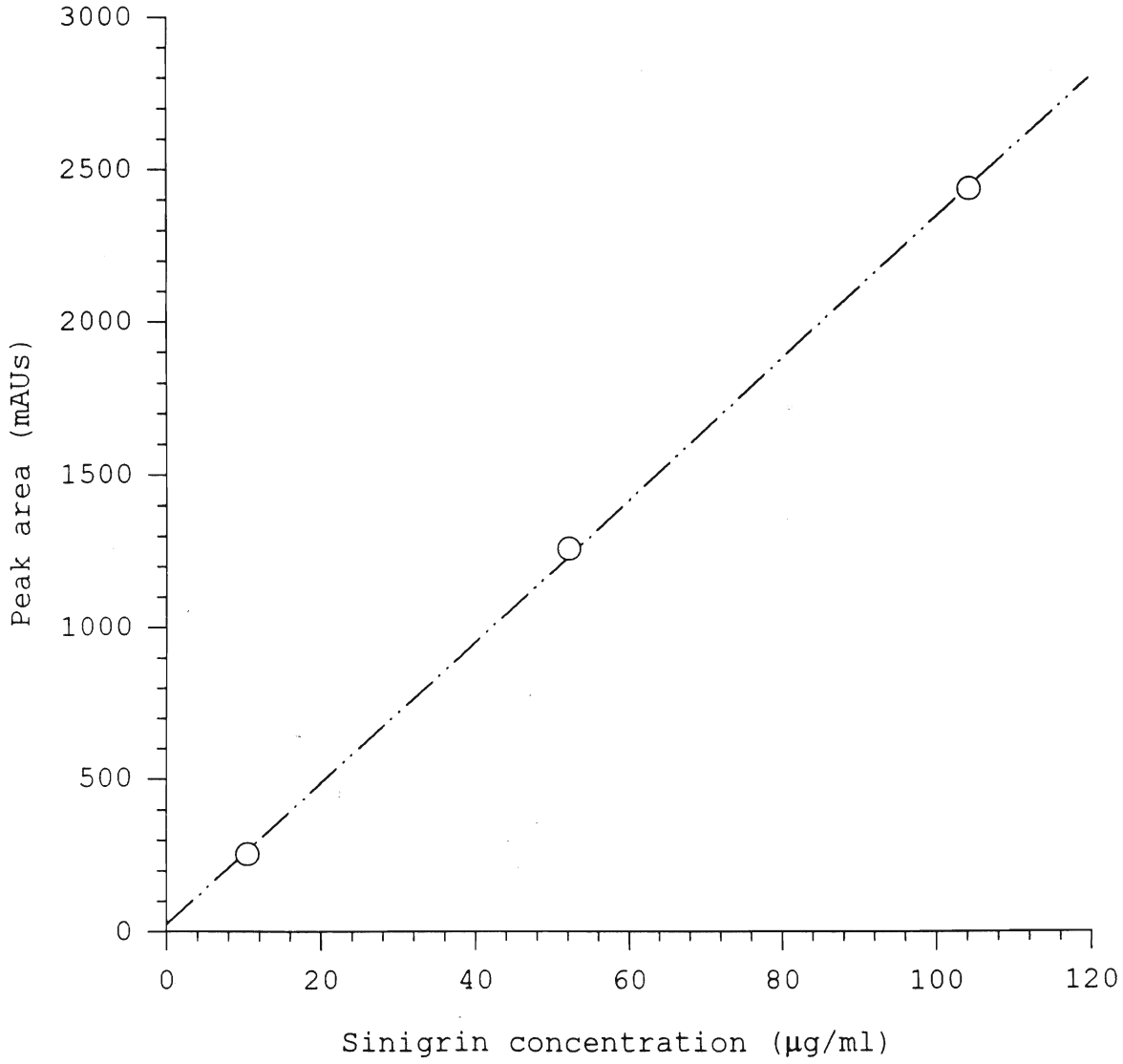
Appendix 1: Root deformity of carrots due to the root-knot nematode (*Meloidogyne hapla*). Two infected carrots are shown on the right. A healthy uninfected carrot is shown on the left.



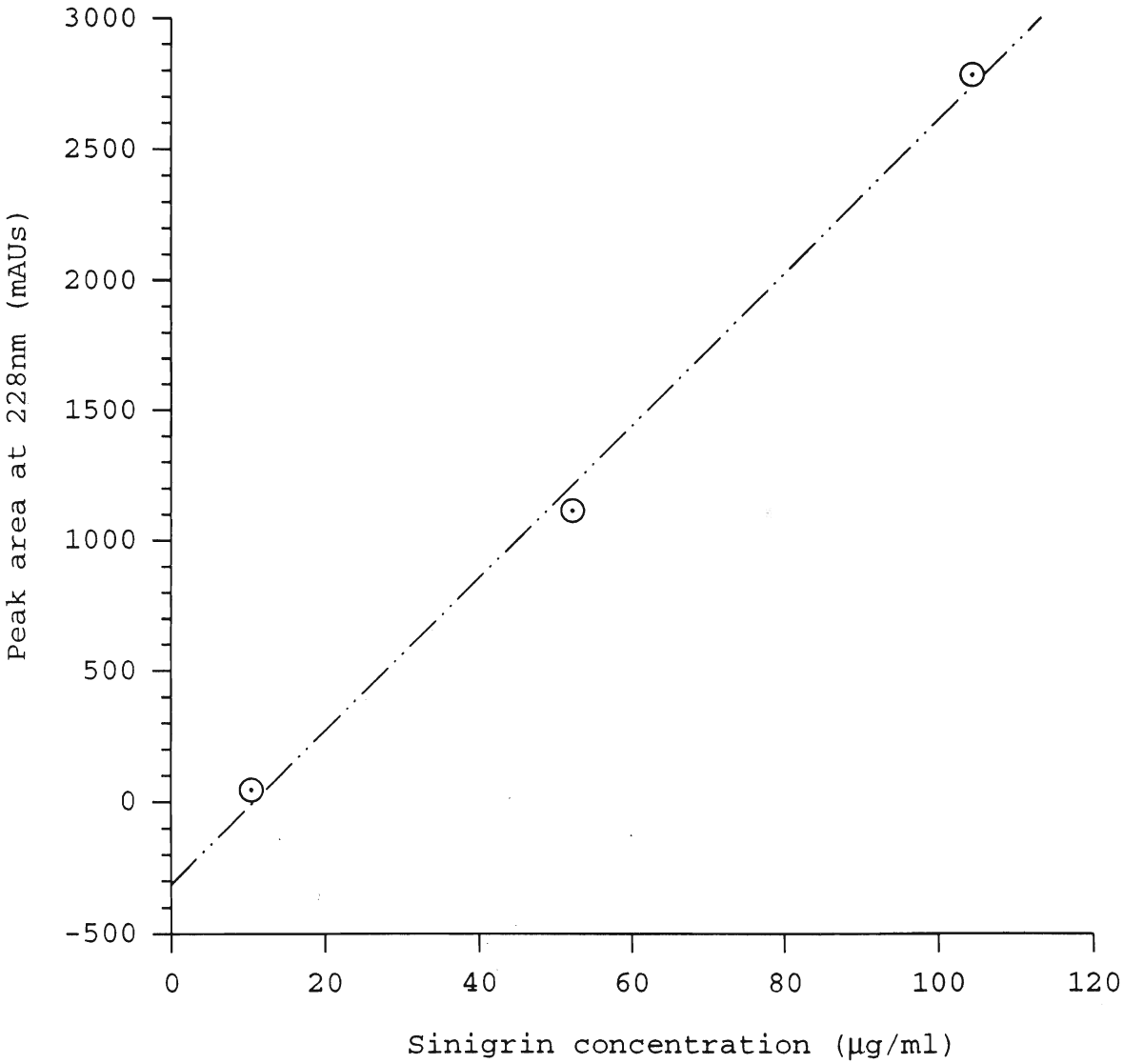
Appendix 2: Linearity of UV detector response for sinigrin at 228nm with an acetate buffer mobile phase



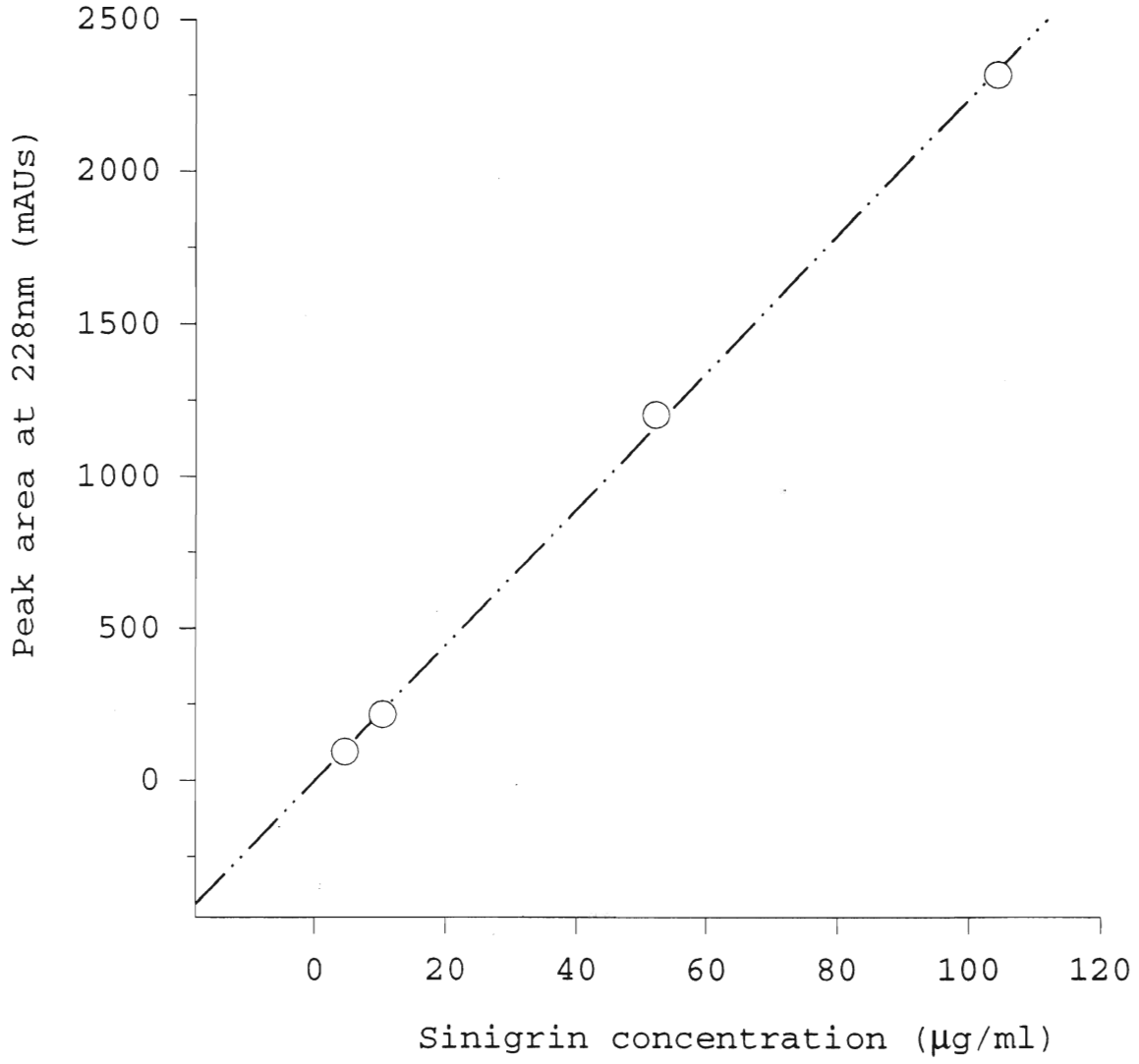
Appendix 3: Linearity of UV detector response for sinigrin at 228nm with a phosphate buffer mobile phase



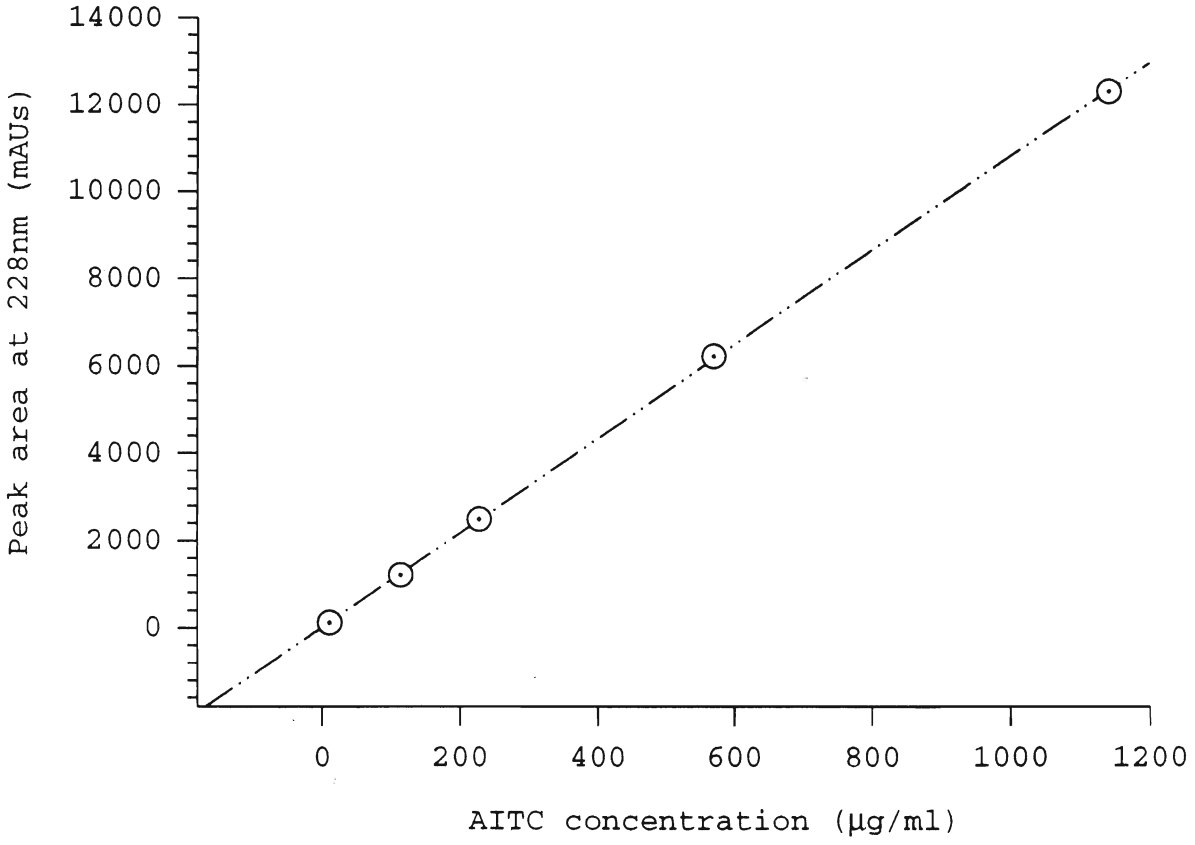
Appendix 4: Linearity of UV detector response for sinigrin at 228nm with an ion-pair mobile phase



Appendix 5: Linearity of UV detector response for sinigrin at 228nm with a phosphate buffer step gradient system

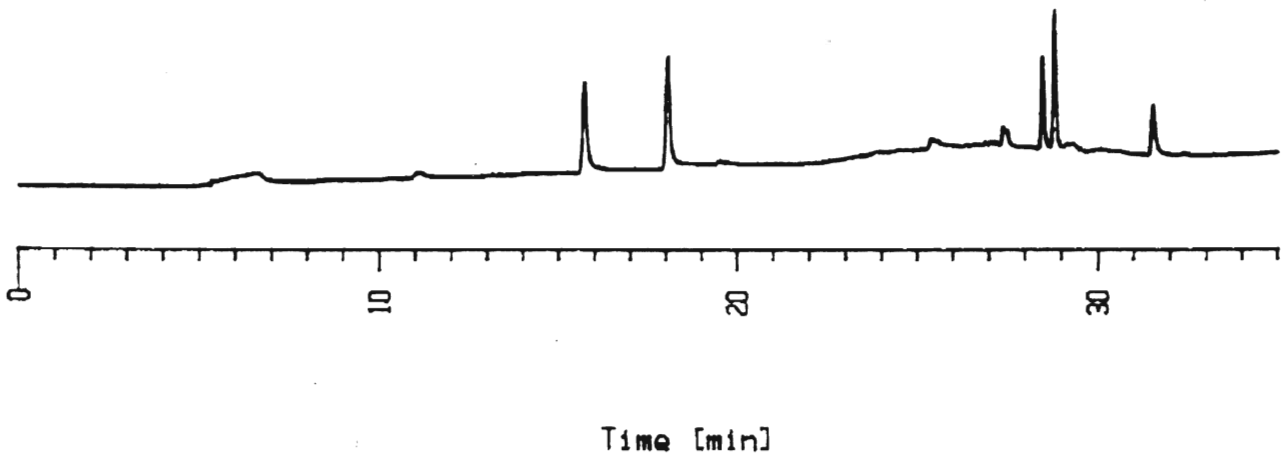


Appendix 6: Linearity of UV detector response for AITC at 228nm with an acetonitrile/water gradient mobile phase system

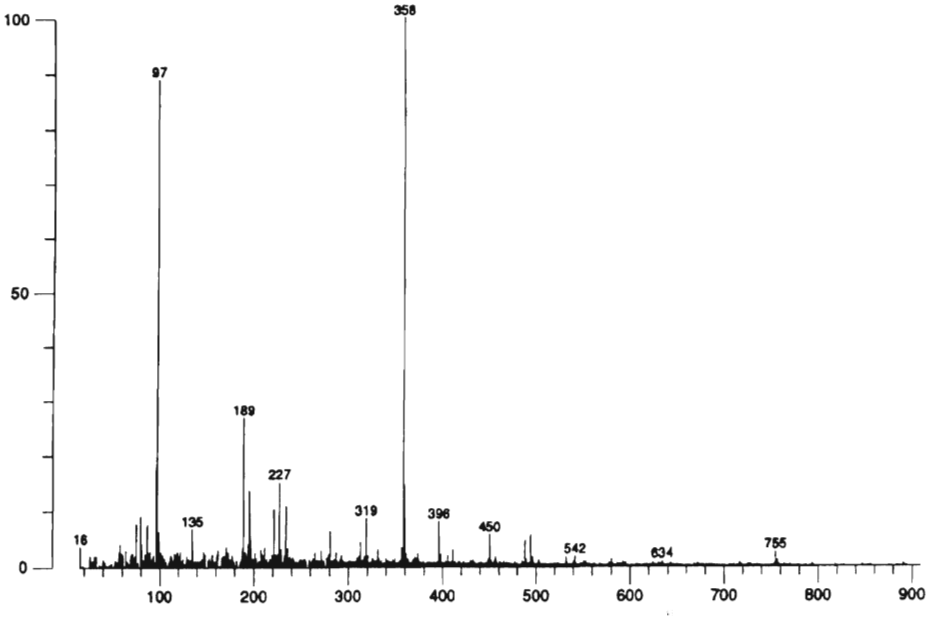


Appendix 7: Acetonitrile/water gradient blank chromatogram for the determination of AITC (200nm)

Detection: UV absorption
at 200nm, full scale
1000mAU

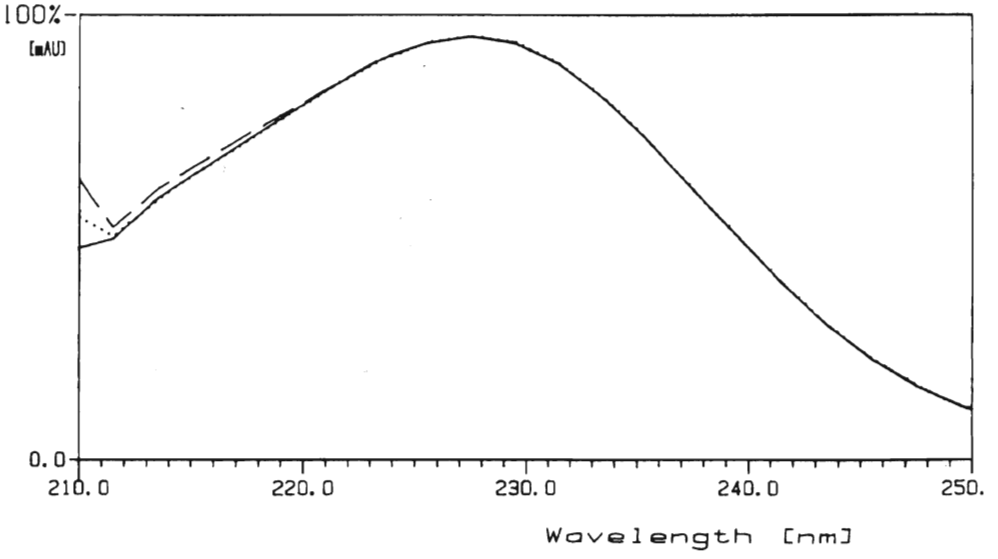


Appendix 8: Spectral data for HPLC standards and AITC degradation compounds



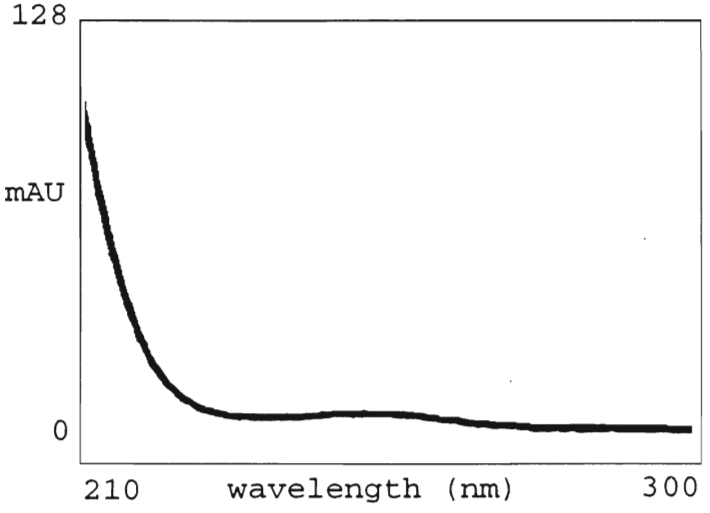
Negative FAB mass spectrum of sinigrin in a glycerol matrix

Full scale 201.9 mAU

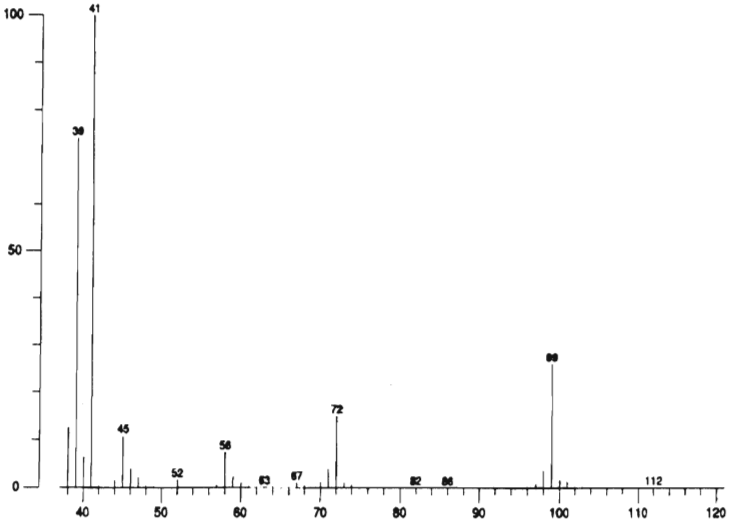


UV Absorption spectrum of sinigrin (25µg)
HPLC separation: Selectosil 5 C18 150x4.6mm analytical column, 45% acetonitrile/55% water 1mM CTAB mobile phase

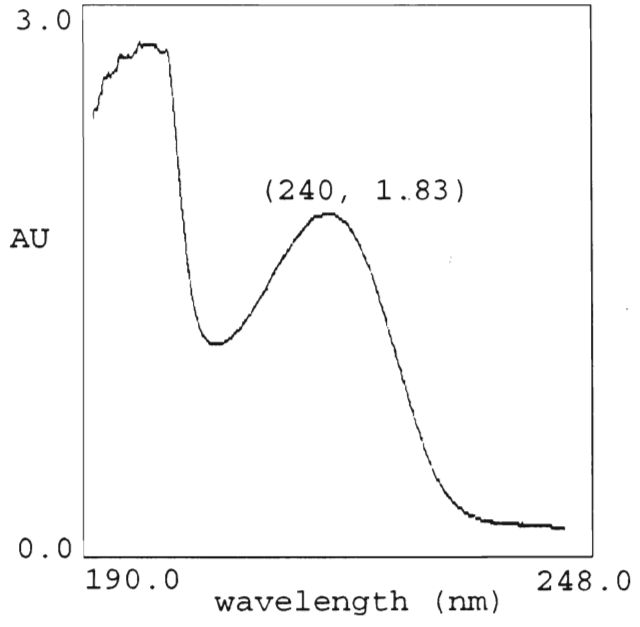
Appendix 8: Spectral data for HPLC standards and AITC degradation compounds



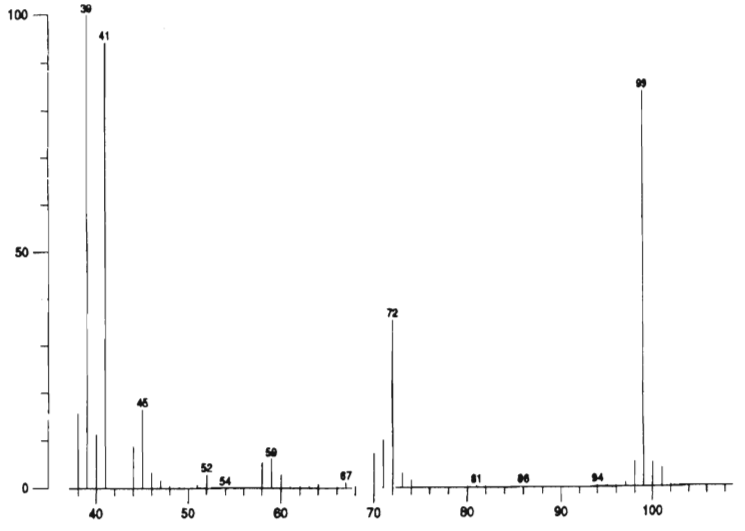
UV absorption spectrum of allyl-thiocyanate. Separated from a 1142 µg/g AITC standard prepared in acetonitrile. The RP-HPLC method previously described was used.



EI mass spectrum of allyl-thiocyanate separated from a sample of AITC. The GC-MS procedure described below was used.

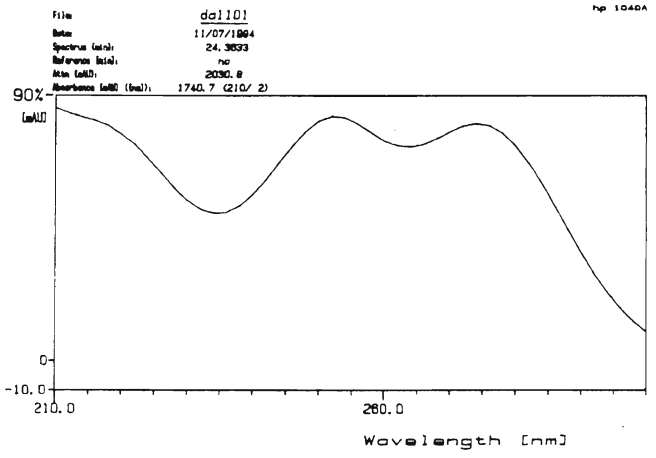


UV absorption spectrum of AITC (359µg/g in water). The DMS-100 UV-Vis spectrophotometer was used.

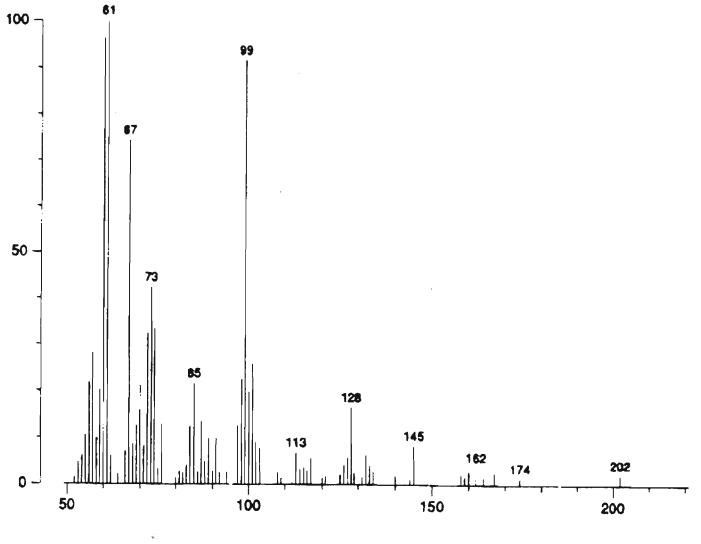


EI mass spectrum of AITC. A direct injection of AITC was separated on a DB-5 column by GC. Temperature of the column was held at 40°C for four minutes then increased 10°C/min. until 200°C. The spectrum was aquired under EI conditions (70eV).

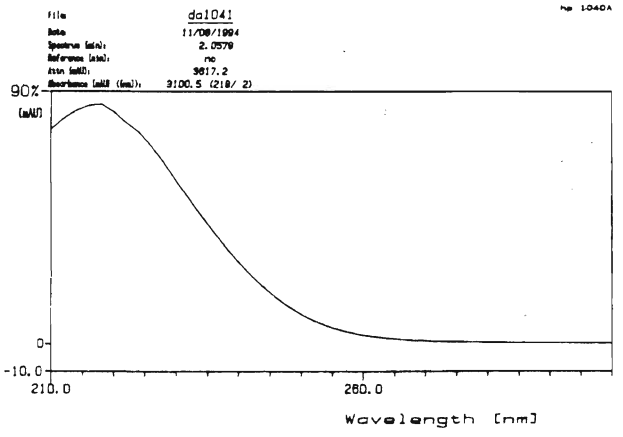
Appendix 8: Spectral data for HPLC standards and AITC degradation compounds



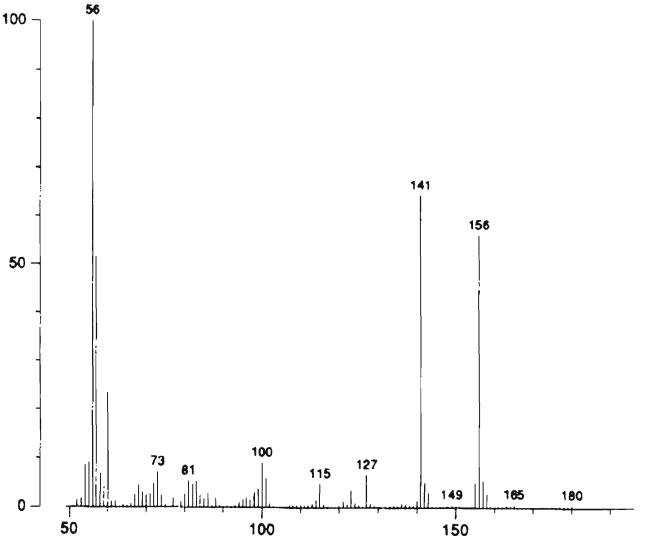
UV absorption spectrum of allyl-allyl-dithiocarbamate (prepared in 30% acetonitrile/water). The RP-HPLC (AITC) method previously described was used.



EI mass spectrum of allyl-allyl-dithiocarbamate (30eV). The sample was introduced by the PB HPLC-MS interface.

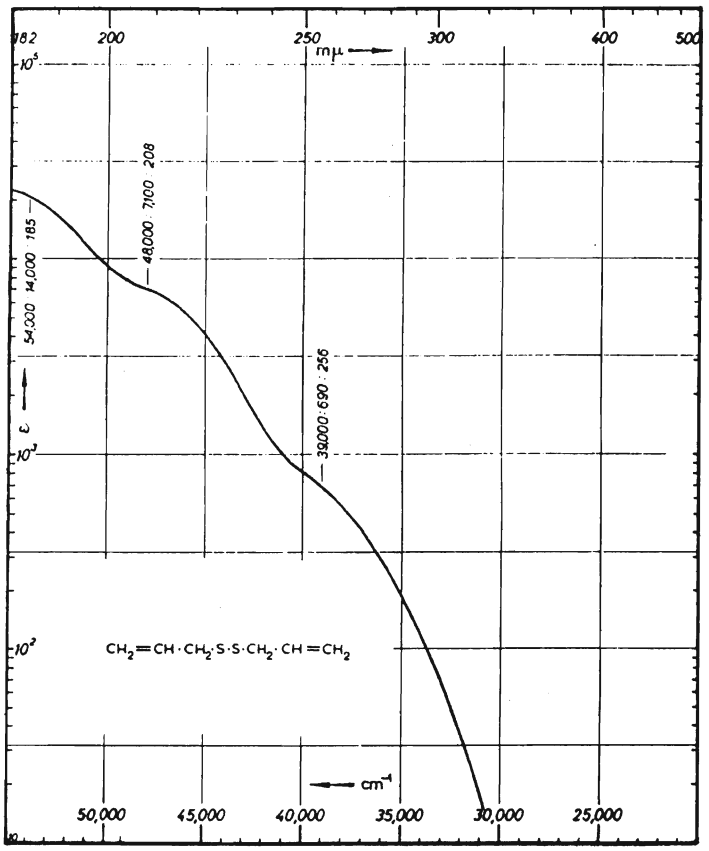


UV absorption spectrum of sodium allyl-dithiocarbamate (30.3 µg). The RP-HPLC (AITC) method previously described was used.

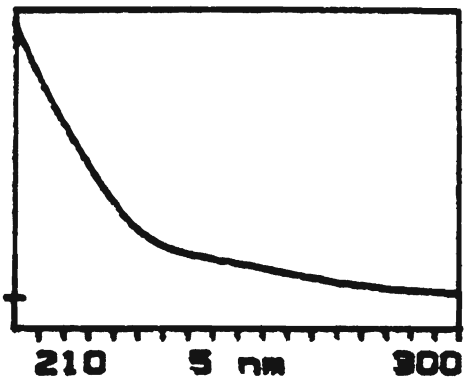


EI mass spectrum of 1,3-diallyl-2-thiourea (70eV). The sample was introduced by the direct probe.

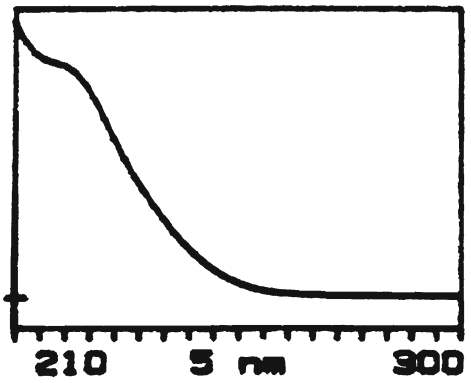
Appendix 8: Spectral data for HPLC standards and AITC degradation compounds



UV absorption spectrum of diallyl-disulfide[243]

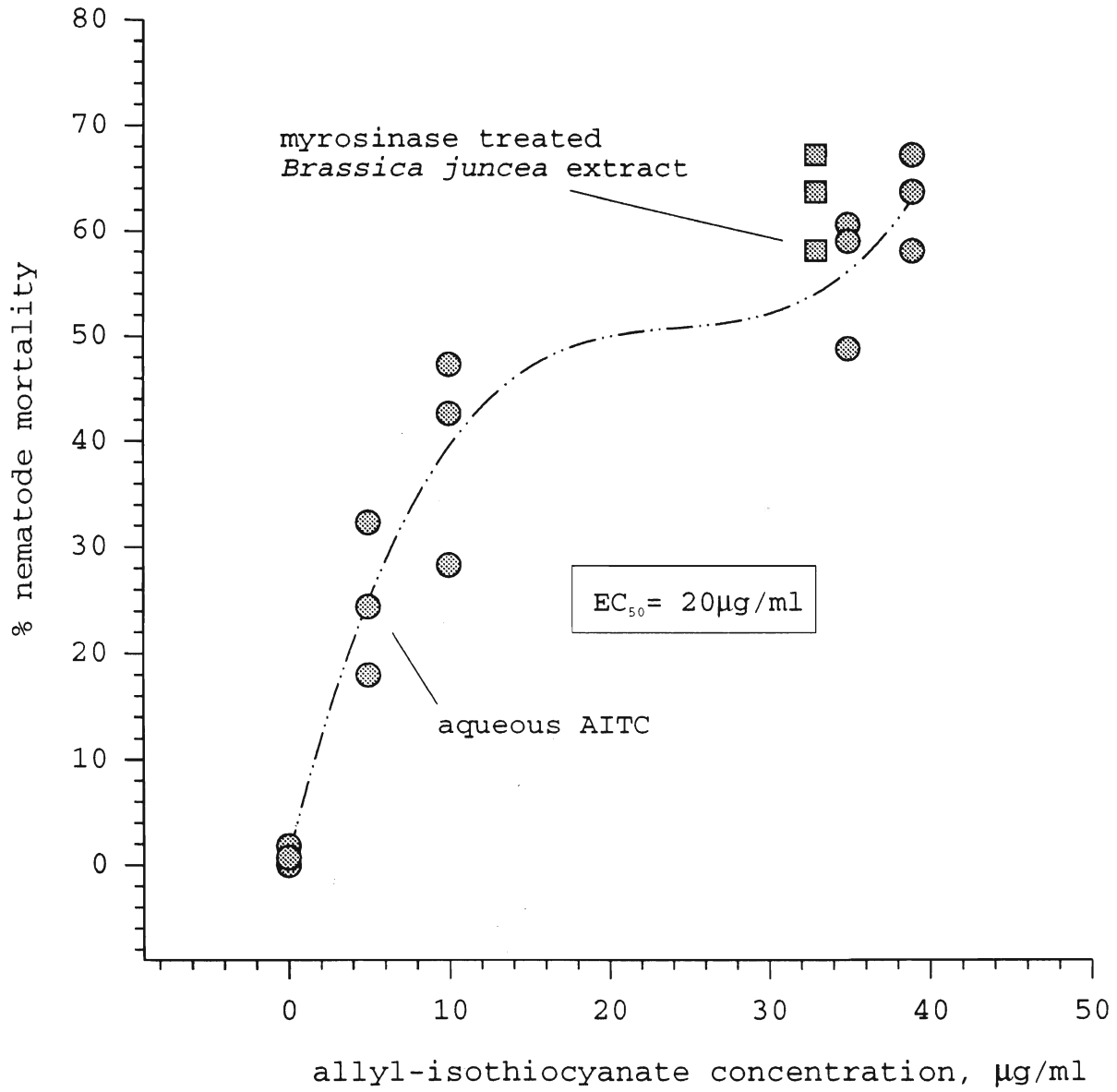


UV absorption spectrum of diallyl-disulfide



UV absorption spectrum of diallyl-sulfide.

Appendix 9: Nematode (*Pratylenchus penetrans*) mortality versus one hour exposure to aqueous AITC



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